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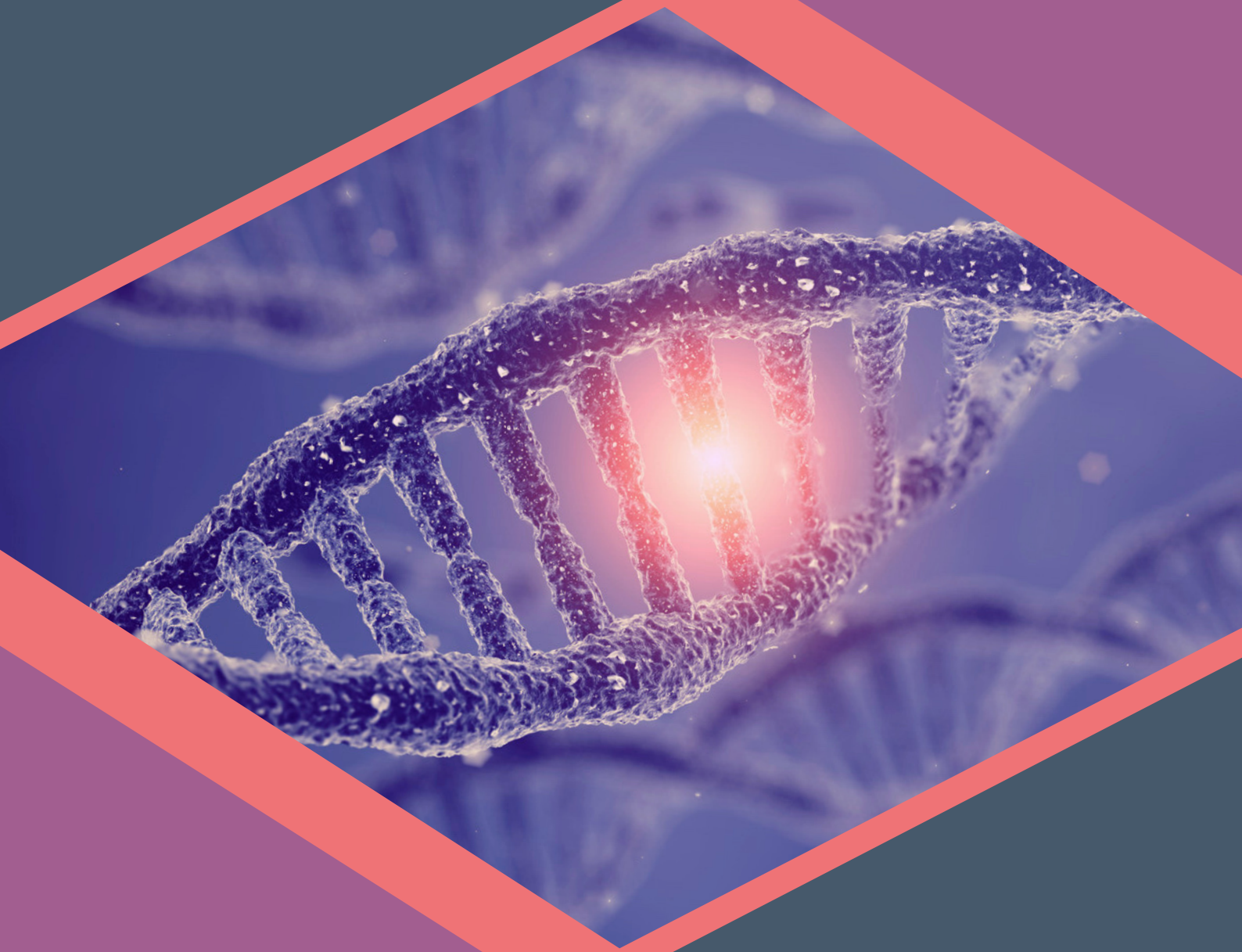

CRISPR

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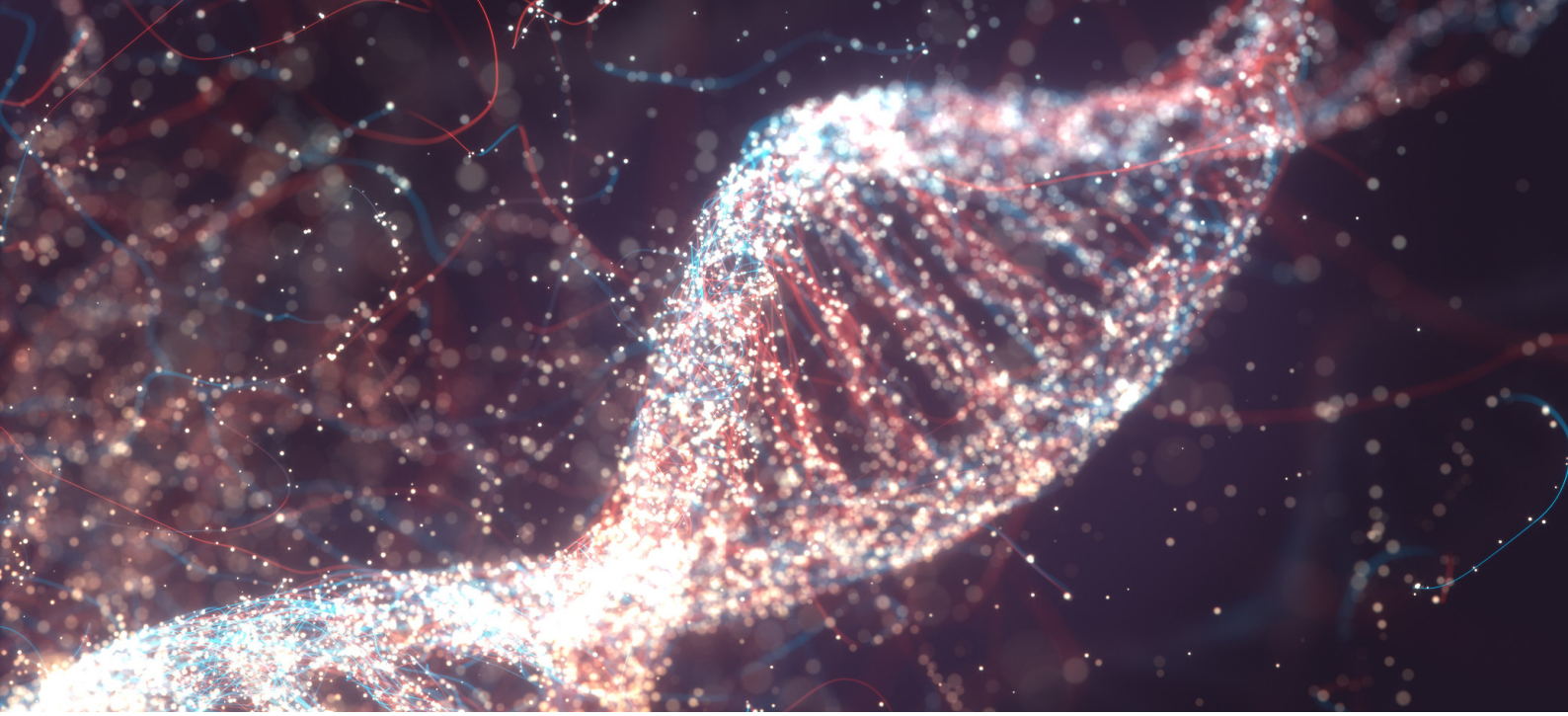

CONTENTS

A peek behind the paper: Pooled CRISPR screens with imaging on microraft arrays reveals stress granule-regulatory factors

Take a look behind the scenes of an article recently published in *Nature Methods* entitled **'Pooled CRISPR screens with imaging on microraft arrays reveals stress granule-regulatory factors'**, as we invite co-First Authors, Emily Wheeler and Anthony Vu, as well as Senior Author, Gene Yeo, to shed some light on their recent research.

Please can you give us a short summary of the method presented in your Nature Methods article, "Pooled CRISPR screens with imaging on microraft arrays reveals stress granule-regulatory factors"?

In this work, we developed an easy-to-use workflow that combines pooled CRISPR screens with high-content imaging and microraft-array technology to screen image-based phenotypes. We named the approach CRaft-ID (CRISPR-based microRaft, followed by gRNA identification). This method is inexpensive, employs standard laboratory equipment, and can be adapted to investigate a wide range of cellular and subcellular phenotypes.

As a proof-of-concept, CRaft-ID was utilized to screen a pool of cells infected with a lentiviral CRISPR library to identify genes that affect the abundance of stress granules. Stress granules are cytoplasmic RNA–protein aggregates that form under stress and are best analyzed using microscopy. This is a challenge for pooled CRISPR screens, which are limited to standard readouts that include survival or fluorescent markers for sorting. In order to perform a pooled CRISPR screen with an imaging readout, we customized the microraft array technology to easily culture thousands of distinct cell colonies in a shared media reservoir. We modified the microrafts by fixing a glass slide to the bottom of the array to enable, for the first time, high-resolution automated confocal microscopy across the entire array. To analyze the hundreds of thousands of microwell images, we developed image processing algorithms to identify colonies with our desired phenotype of reduced stress granule formation. Microrafts containing these colonies were then isolated, allowing us to identify and validate known and novel factors that affect the abundance of stress granules. This highlights the applicability of our approach to facilitate image-based pooled CRISPR screens.

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Pooled CRISPR screens with imaging on microraft arrays reveals stress granule-regulatory factors

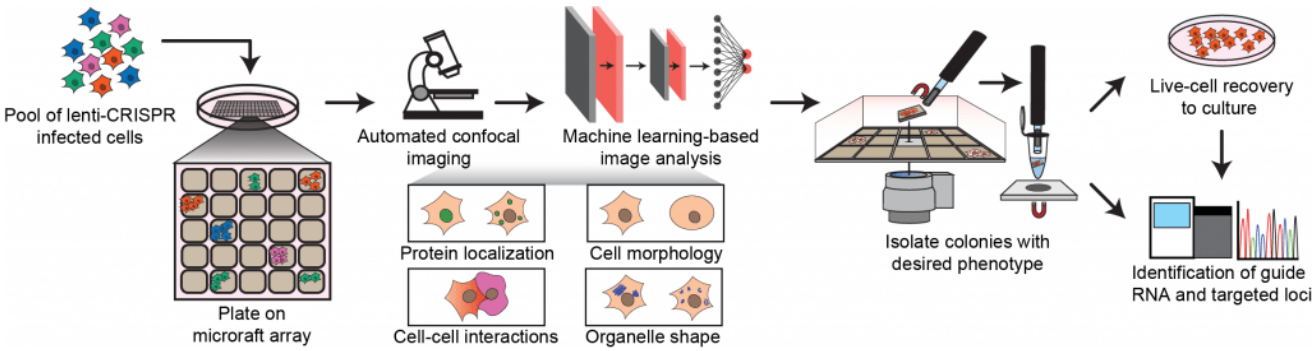

Figure 1. The CRaft-ID workflow.

What scientific problem inspired you to develop this new method?

Pooled CRISPR screens have transformed the life science community as an extremely powerful tool to identify genetic factors that are critical for a selected phenotype. Conveniently, this approach allows us to deplete thousands of genes simultaneously in a single sample, but until now we have been limited in microscopic visualization of that pool of cells. By modifying the microraft arrays with a glass back, we demonstrate a high-throughput method to screen a pool of genetically modified cells by an image-based phenotype, and importantly, provide a means to easily isolate colonies of interest for follow up studies.

What impact do you hope it will have on laboratory researchers?

CRaft-ID will provide an inexpensive and accessible means for researchers to easily perform image-based pooled CRISPR screens in any standard laboratory setting. In contrast to current image-based screens that are performed in array format, our workflow does not require specialized robotic equipment for the manipulation of thousands of individual wells. The flexible platform is compatible with any CRISPR library, a variety of cell types, and countless cellular or subcellular phenotypes and therefore can be easily adapted by any researcher wanting to systematically annotate gene function with their phenotype of interest.

The technique appears relatively simple, with the software freely available online. Do you have any tips or tricks for others looking to utilize this method?

The microraft arrays behave like standard tissue culture plates, and therefore can accompany a wide range of cell types. However, nondividing cells are not suitable for direct plating onto the platform, where colonies are required for imaging and genomic DNA isolation. For such cases, we recommend users plate stem cells or intermediates with proliferating cells onto microraft and continue differentiation after colony formation.

Given that the penetrance of genome editing in cells transduced with lentiCRISPR guides can be highly variable, high coverage of a pooled CRISPR screen is recommended to increase the likelihood of capturing a knock-out for a selected phenotype. In our screen, we designed 10 unique

sgRNAs targeting each gene and screened 10x as many cells as guides in the library, resulting in ~100 opportunities to phenotype each gene in the library. With this coverage, we are able to identify both novel and known genetic regulators of stress granule abundance. However, if higher coverage is desired, users should employ a smaller, focused library of sgRNAs or include more microraft arrays for imaging.

Before performing a screen with CRaft-ID, please read through this detailed step-by-step experimental protocol available online.

Critical steps in the protocol include:

- Centrifuging cells plated on microrafts in a swinging-bucket centrifuge to minimize the likelihood of cells adhering to microwell walls, resulting in out-of-focus colonies when imaging.
- Before plating onto microrafts: thoroughly dissociate trypsinized cells by repeated pipetting, strain the cell suspension, and plate at recommended cell density to reduce colony doublet formation.
- After isolating microwell with target cell colony, immediately proceed to DNA extraction with Quick Extract Buffer prior to storage at -20°C to increase the efficiency of genomic DNA extraction.
- Perform DNA extraction and first PCR reaction in pre-AMP PCR clean space to avoid library contamination.

What equipment would a researcher looking to utilize your method need to have to hand?

The CRaft-ID workflow was designed to be widely accessible by employing standard laboratory equipment, CRISPR libraries that do not require modifications, and commercially available materials. Microraft arrays can be purchased through Cell Microsystems (NC, USA) and will require the simple modification of securing a 1-mm-thick glass slide to the bottom of the array before imaging on any standard automated confocal microscope. Our CRaft-ID software used to process and analyze the acquired images is available in an open-access GitHub repository and can be executed on any conventional workstation. Finally, users will need a motorized microneedle device and a hand-manipulated magnetic wand to isolate the magnetized microrafts containing candidate cells for PCR-based sequencing. Both the microneedle device and magnetic wand are commercially available through Cell Microsystems.

What are you hoping to do next in this area?

After successfully identifying and validating novel and known regulators of stress granule formation, we will use CRaft-ID to discover genes critical for resolving stress granules after stress removal. Defects in stress granule assembly and clearance are firmly linked to neurodegenerative diseases and cancer, and therefore identification of genes essential to stress granule dynamics could reveal novel disease-relevant therapeutic targets. A unique feature of our image-based phenotypic screen is the ability to perform live-cell imaging of fluorescently labeled subcellular proteins followed by live-cell capture. By using an engineered cell line expressing a fluorescently labeled stress granule protein, we can now visualize real-time dynamics of stress granule behavior from our pool of lentiCRISPR-infected cells.

Pooled CRISPR screens with imaging on microraft arrays reveals stress granule-regulatory factors

We also hope to expand the utility of CRaft-ID to other measurable subcellular phenotypes, including intracellular-RNA localization. Spatial and temporal regulation of RNA is critical for many cellular functions including cell motility, polarization and development, and therefore we aim to use our platform to determine which RNA-binding proteins are involved in the localization of mRNA transcripts critical for biological processes. Our approach is broadly applicable to many settings and we are excited to use it across new phenotypes that can be analyzed by advanced-microscopy technology and image-analysis algorithms.

Gene Yeo is a Professor of Cellular and Molecular Medicine at the University of California San Diego (UCSD; CA, USA), a founding member of the Institute for Genomic Medicine and member of the UCSD Stem Cell Program and Moores Cancer Center. Yeo has a BSc in Chemical Engineering and a BA in Economics from the University of Illinois, Urbana-Champaign (USA), a PhD in Computational Neuroscience from Massachusetts Institute of Technology (USA) and an MBA from the UCSD Rady School of Management. He is a computational and experimental scientist who has contributed to RNA biology and therapeutics. His primary research interest is in understanding the importance of RNA processing and the roles that RNA binding proteins (RBPs) play in development and disease.

Anthony Vu is a PhD student in UCSD's Biomedical Sciences program. Prior to joining the program, Vu received a joint BS/MS degree in Biology from UCSD where he completed his master's thesis work in Fred Gage's lab at the Salk Institute (CA, USA) studying the molecular mechanisms by which critical neuron-specific gene repressor REST regulates neuronal development. In the Yeo lab, Vu is currently developing high-throughput genetic screens to study the mechanisms of protein aggregation underlying neurodegenerative disease to identify potential therapeutic targets. He is also interested in designing new tools to characterize the spatial and temporal regulation of RNA-protein complexes critical for neuronal function and health. Vu is a NSF GRFP fellow and an ARCS scholar since 2019.

Emily Wheeler received her PhD in Biomedical Sciences from UCSD. Her thesis work in Yeo's lab focused on using computational and molecular biology tools to uncover how protein—RNA interactions in cells play a role in cancer and neurodegeneration. Specifically, her work included uncovering novel RNA-binding proteins that are essential to form protein aggregates in motor neurons that cause cell death in amyotrophic lateral sclerosis. Additionally, she developed computational methods to determine how point mutations in splicing factors that occur in blood cancer alter their interaction with cellular RNA. She is a NSF GRFP fellow and an ARCS scholar since 2017. More recently, Wheeler joined Zuzana Tothova's lab at the Harvard Medical School (MA, USA) as a postdoctoral fellow where she is studying the role of mutations in cohesion proteins to drive clonal hematopoiesis and blood cancer.

Wheeler EC, Vu AQ, Einstein JM *et al*. Pooled CRISPR screens with imaging on microraft arrays reveals stress granule-regulatory factors. *Nat. Methods* 17, 636–642 doi: 10.1038/s41592-020-0826-8 (2020).

A peek behind the paper: Rebeca Carballar-Lejarazú on digital droplet PCR and IDAA for the detection of CRISPR indel edits in *Anopheles stephensi*

Take a look behind the scenes of a recent reports article published in *BioTechniques* entitled '**Digital droplet PCR and IDAA for the detection of CRISPR indel edits in the malaria species *Anopheles stephensi***', as we ask author, Rebeca Carballar-Lejarazú from the University of California, Irvine (USA) about the use of these two novel techniques and how they can be effectively used to detect resistance alleles in large populations of mosquitoes over many generations.

Please can you give us a short summary of the recent reports article, 'Digital droplet PCR and IDAA for the detection of CRISPR indel edits in the malaria species Anopheles stephensi'?

Mosquito-borne diseases represent a major problem in human health; unfortunately the existing vector control strategies are insufficient to control those diseases. New genetic technologies, such as CRISPR/Cas9 systems, have transformed the field of genome modification and are considered a promising tool for combating malaria and other mosquito-borne diseases.

Recent progress demonstrated that CRISPR/Cas9 gene-drive derived systems drive target-specific gene conversion at ≥99.5% efficiency in transgene heterozygotes of *Anopheles stephensi* AsMCRkh2 line (which was used in this study), however, the gene drive efficiency depends on cleaving the target version (often wild-type) of a gene and promoting the cell repair mechanisms to copy an engineered version of the gene (containing the gene drive) into the damaged version.

Alternatively, during the repair process, the generation of resistance alleles to the drive can be observed. Accumulation of resistance alleles can lead to diminishing effect on the drive thereby mosquito progeny will go back to the Mendelian inheritance. There are several methods to detect resistance alleles, however, none of those methods are suitable for high-throughput screening of resistance alleles in samples from multiple, large cage populations or field trials due to their technical complexity, cost, time or labor. Here we report the use of two novel techniques (digital droplet PCR (ddPCR) and indel detection by amplicon analysis (IDAA)) that can be effectively used to detect resistance alleles in large populations of mosquitoes over many generations.

BioTechniques
The International Journal of Life Science Methods

Digital droplet PCR and IDAA for the detection of CRISPR indel edits in *Anopheles stephensi*

What led you to carry out this research?

There has been considerable discussion and debate on the impact of drive-resistant alleles and their impact on the efficiency of gene-drive systems. Resistant alleles may either be pre-existing in the wild mosquito populations or can be generated as a product during the drive. Mosquito genomes are highly polymorphic in natural populations and therefore potentially disposed to have pre-existing resistance alleles. High-throughput screening in mosquito populations can contribute not only to determine the variability of a desired target site but also to determine, in a quantitative way, the accumulation of resistance alleles in populations carrying drive systems.

What were the key conclusions; what impact do you foresee this research having?

This will contribute to a more sensitive and reproducible detection of resistance alleles in mosquito samples and a more efficient analysis of indel quantification in a cost and time saving manner. Moreover, these high throughput – yet cost-effective – methods can provide the basis for large screenings in cage trials and field trials during the process for deploying gene drive in mosquito populations into the field.

What work are you hoping to do next in this area?

I am currently working on a next-generation gene drive for the African malaria vector *Anopheles gambie* that shows low frequency of resistance alleles and a very robust drive efficiency. We are coupling this drive to some effector genes that will make our mosquitoes resistant to the malaria parasite. We expect that this new system can be deployed into the field to contribute to the malaria control together with the pre-existent strategies.

Rebeca Carballar-Lejarazú is a Senior Scientist of Microbiology & Molecular Genetics. She is a pioneer on mosquito gene drives and its application for malaria control. She has experience in insect molecular genetics, vector biology and the development of insect genetic technologies. She works in fields related to synthetic molecular biology, genetics, gene drive, and vector biology to develop genetic control strategies against mosquitoes.

She is currently working on the development and optimization of a gene drive system based on CRISPR/Cas9 technology for population modification of the malaria African vector *Anopheles gambiae* to spread beneficial genes quickly through mosquito populations.

Carballar-Lejarazú R, Kelsey A, Binh Pham T, Bennett EP & James AA. Digital droplet PCR and IDAA for the detection of CRISPR indel edits in the malaria species *Anopheles stephensi*. *BioTechniques* 68 (4), doi:10.2144/btn-2019-0103 (2020).

Digital droplet PCR and IDAA for the detection of CRISPR indel edits in the malaria species *Anopheles stephensi*

Rebeca Carballar-Lejarazú*[,1], Adam Kelsey[1], Thai Binh Pham[1], Eric P Bennett[2] & Anthony A James[1,3]

ABSTRACT

CRISPR/Cas9 technology is a powerful tool for the design of gene-drive systems to control and/or modify mosquito vector populations; however, CRISPR/Cas9-mediated nonhomologous end joining mutations can have an important impact on generating alleles resistant to the drive and thus on drive efficiency. We demonstrate and compare the insertions or deletions (indels) detection capabilities of two techniques in the malaria vector mosquito *Anopheles stephensi*: Indel Detection by Amplicon Analysis (IDAA™) and Droplet Digital™ PCR (ddPCR™). Both techniques showed accuracy and reproducibility for indel frequencies across mosquito samples containing different ratios of indels of various sizes. Moreover, these techniques have advantages that make them potentially better suited for high-throughput nonhomologous end joining analysis in cage trials and contained field testing of gene-drive mosquitoes.

METHOD SUMMARY

Mosquito DNA was extracted with the Promega Wizard® Genomic DNA Purification Kit protocol and quantified with Qubit® 3.0 following manufacturer protocols. PCR products for IDAA and ddPCR were generated with primers spanning 150–500 bp around the target site. IDAA amplicons were sent directly to COBO Technologies for analysis. ddPCR amplicons were analyzed using the Bio-Rad QX200™ ddPCR system.

KEYWORDS

CRISPR-Cas9 • ddPCR • gene editing • IDAA • mosquitoes • NHEJ quantification

[1]*Department of Microbiology & Molecular Genetics, University of California, Irvine, CA 92697-4025, USA;* [2]*Department of Odontology, Copenhagen Center for Glycomics, Faculty of Health Sciences, University of Copenhagen, Copenhagen DK-2200, Denmark;* [3]*Department of Molecular Biology & Biochemistry, University of California, Irvine, CA 92697-3900, USA;* **Author for correspondence: rcarball@uci.edu*

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CRISPR/Cas9 gene-editing technology has transformed the field of genome modification. This system is composed of two fundamental components that interact to form a complex: Cas9 endonuclease and sgRNA, a target-specific RNA that guides Cas9 to the desired genomic DNA target site. Cas9 induces a double strand break at the target site, activating the DNA repair pathways of homology-directed repair (HDR) and nonhomologous end joining (NHEJ). HDR can induce accurate gene repair of one to thousands of base pairs in the presence of a homologous donor molecule, allowing for the correction of point mutations and introduction of exogenous sequences. In contrast, NHEJ produces genetic lesions comprised of random sizes of small insertions or deletions (indels) that alter the target site and can disrupt gene function. The HDR mechanism offers the opportunity to genetically modify large populations of arthropods, among other model organisms, by integrating the *Cas9* endonuclease gene, the sgRNA targeting the desired locus and a dominant marker (fluorescent protein). The cassette is autonomous and can replicate to the homologous chromosome through HDR. This process effectively converts a heterozygous organism into a homozygote for the desired synthetic cassette, resulting in a selfish pattern of inheritance [1]. The nature of this type of genetic modification is designated gene drive and has been proposed as a tool for genetically modifying mosquito populations [2,3].

Gene drive in mosquitoes has been proposed as a promising tool for combating malaria and other mosquito-borne diseases, including dengue and zika [4], either by population suppression by spreading a lethal gene in wild-type (WT) mosquito populations to cause population crash or by replacement through the introduction of an anti-pathogen gene into a WT population. Recent progress demonstrated that CRISPR/Cas9 gene-drive-derived systems drive target-specific gene conversion at ≥99.5% efficiency in transgene heterozygotes of the *Anopheles stephensi* AsMCRkh2 line [5]. Gene drive efficiency depends on the availability of WT or susceptible alleles targeted by the gRNA-directed Cas9 cleavage. When a susceptible chromosome has been mutated by NHEJ, the key nucleotides necessary for gRNA recognition could be mutated or eliminated, thus preventing subsequent HDR-mediated gene conversion in the mosquito germline. An accumulation of NHEJ events has a diminishing effect on the drive, and the mosquito progeny approach Mendelian inheritance of the introduced DNAs due to the generation of drive-resistant loci [5,6]. Methods to detect NHEJ events rely on artificial reporter assays, gel-based systems, Sanger sequencing and deep sequencing [7–9]. None of these methods is suitable for high-throughput screening of NHEJ alleles in samples from multiple, large-cage populations or field trials due to their technical complexity, cost and time or labor required. A resistant Cas9-induced NHEJ allele percentage is considered acceptable when it is lower than the naturally occurring single nucleotide polymorphisms (SNPs) at the target site in the wild population [10]. This percentage can be tolerated while not affecting drive fixation; therefore, NHEJ quantification is an essential parameter during laboratory and field trials. Detecting indels in large populations of mosquitoes over many generations requires a high-throughput method that maximizes efficiency and provides

sensitive, accurate results. To circumvent the difficulties of conventional techniques, we compared two novel techniques, Droplet Digital PCR™ (ddPCR™; Bio-Rad Laboratories, CA, USA) and Indel Detection by Amplicon Analysis (IDAA™; COBO Technologies, Copenhagen, Denmark) for NHEJ quantification in the *A. stephensi* AsMCRkh2 line carrying a CRISPR/Cas9 gene drive.

MATERIALS & METHODS

Sample sources

A. stephensi mosquitoes (Indian strain, gift of M. Jacobs-Lorena, Johns Hopkins University) maintained at the University of California, Irvine (UCI) insectary are the source of all insects used in the experiments. The gene-drive line AsMCRkh2 (gene drive) and WT (non-gene drive) mosquitoes were maintained at 27°C with 77% humidity and a 12-h day/night, 30-min dusk/dawn lighting cycle. AsMCRkh2 mosquitoes with indels were recovered from crosses between WT and AsMCRkh2 mosquitoes over 20 generations [11]. The Cas9-targeted sequence, 5′-GATGGTTCCGTTCTACGGGCAGG-3′ (protospacer adjacent motif sequence underlined), is in the gene encoding *kynurenine hydroxylase* (*kh*).

DNA extraction & quantification

Genomic DNA extraction was performed using the Wizard® Genomic DNA Purification Kit protocol (Promega, WI, USA) for mouse tails according to the manufacturer's instructions. Pools of 10 adult mosquitoes were used for DNA extraction. DNA was resuspended in 50 µl of PCR-grade water. DNA extracts were quantified at the UCI Genomics High-Throughput Facility using a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific, MA, USA) following the manufacturer's instructions. One microliter of DNA extract was analyzed using the Qubit dsDNA HS Assay Kit followed by Qubit 3.0 quantification.

ddPCR drop-off assay

We prepared 25-µl reactions with 12.5 µl Bio-Rad ddPCR 2× Supermix for Probes (No dUTP), 10 µl DNA (0.9 ng/µl), 1.25 µl fluorescein amidite (FAM)/forward (5-µM FAM probe, 18-µM forward primer) (Supplementary Table 1) and 1.25 µl hexachlorofluorescein (HEX)/reverse (5 µM HEX probe, 18-µM reverse primer) (Supplementary Table 1) in a 96-well PCR plate. Twenty microliters from the PCR reactions were used for droplet generation, each theoretically containing 30,000 haploid genome copies per 20-µl reaction, assuming that one *A. stephensi* haploid genome is 0.24 pg [12]. Droplets were generated at the UCI Genomics High-Throughput Facility using a Bio-Rad QX200 Droplet Generator following the manufacturer's instructions; they were then transferred to a Bio-Rad 96-well PCR plate and foil

Table 1. Insertions or deletions quantification in nonhomologous end joining mosquito samples from small-cage trials of the gene drive AsMCRkh2 strain.

Number	Sample (cage name-generation)	ddPCR average indel (%)	IDAA average indel (%)
Indel-1	A1-G3	100.00	100.00
Indel-2	A1-G8	99.97	100.00
Indel-3	A1-G14	100.00	100.00
Indel-4	A1-G16	100.00	100.00
Indel-5	A3-G4	100.00	100.00
Indel-6	A3-G7	100.00	100.00
Indel-7	A3-G8	100.00	100.00
Indel-8	A3-G9	100.00	99.20
Indel-9	A3-G10	100.00	100.00
Indel-10	B1-G4	100.00	100.00
Indel-11	B1-G7	99.97	100.00
Indel-12	B1-G9	100.00	100.00
Indel-13	B1-G10	99.80	100.00
Indel-14	C1-G8	100.00	100.00
Indel-15	C1-G11	99.97	100.00

A total of 15 sample pools of ten mosquitoes each were obtained from different cages through several generations [11]. ddPCR and IDAA were used to analyze the same DNA extract of each sample to quantify the total percentage of indel sequences. Analysis was carried out in triplicate (n = 3) with averages shown. The Pearson correlation coefficient is $r = 0.77$ when comparing similarity trends. Student's t tests performed for each individual sample yielded no statistical significance ($p < 0.05$) between the results of both techniques. IDAA and ddPCR are sensitive in detecting multiple types of indels in a pool sample, as there is no significant difference between the results of the two methods and the expected percentage of indel, which is 100% in all samples. Cage numbers refer to those in Pham *et al.* [11].
ddPCR: Droplet Digital PCR; IDAA: Indel detection by amplicon analysis; Indel: Insertions or deletion.

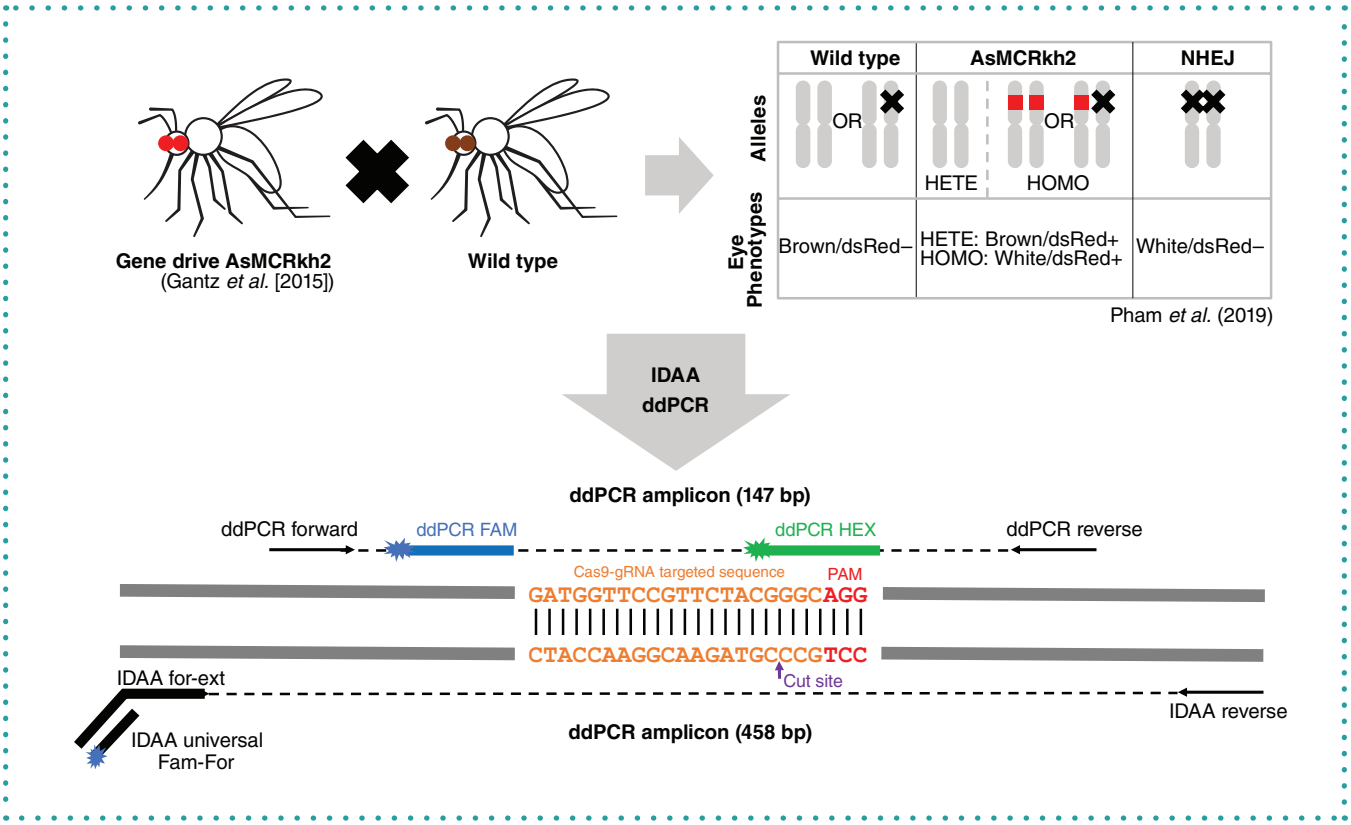

Figure 1. Assessment of nonhomologous end joining alleles from AsMCRkh2 mosquitoes using ddPCR and IDAA techniques. AsMCRkh2 is a gene drive transgenic *Anopheles stephensi* mosquito line that contains an autonomous Cas9-gRNA system linked to a dominant DsRed eye marker that targets the kynurenine hydroxylase (*kh*) locus [5]. WT mosquitoes and heterozygous AsMCRkh2 mosquitoes with one intact *kh* allele have a black-eyed/DsRed-positive phenotype, whereas homozygous AsMCRkh2 individuals have a recessive white-eyed/DsRed-positive phenotype. In contrast, mosquitoes presenting both resistant alleles present a white-eyed/DsRed-negative phenotype. NHEJ mosquitoes used in this report came from cage trials established from an outcross of AsMCRkh2 mosquitoes with WT where a susceptible *kh* allele was cleaved by gRNA-guided Cas9 nuclease and was repaired with NHEJ instead of HDR [5,11] to become a Cas9-resistant *kh* allele with mutations around the cut site. NHEJ mosquitoes from different cage generations were used for DNA extraction and PCR using primers designed to amplify a 458-bp PCR fragment and 147-bp PRC fragment spanning the targeted sequence for IDAA and ddPCR analysis, respectively.
ddPCR: Droplet Digital PCR; HDR: Homology-directed repair; IDAA: Indel detection by amplicon analysis; NHEJ: Nonhomologous end joining; WT: Wild-type.

heat-sealed at 180°C for 5 s. PCR was performed using a Bio-Rad C1000 Touch™ thermal cycler with a 96-deep-well reaction module under the following conditions: 95°C for 10 min, 40 cycles of 94°C for 30 s, 55°C for 1 min and 60°C for 2 min, followed by 98°C for 10 min and a 4°C hold. A 2°C/s ramp rate was used for all steps. Droplets were read using the Bio-Rad QX200 ddPCR system. The data analysis was performed using Bio-Rad QuantaSoft™ Analysis Pro version 1.0.596 in drop-off mode requiring manual cluster designation.

IDAA assay

Samples were prepared from 25-µl PCR reactions using 0.5 U of TEMPase (Amplicon, Odense, Denmark) in 1× ammonium buffer with 2.5-mM $MgCl_2$, 200-µM dNTP, 5% DMSO, 0.25-µM Universal FamFor, 0.025-µM forward-extension primer and 0.25-µM reverse-extension primer (Supplementary Table 1). PCR conditions included an initial incubation at 95°C for 15 min; 15 cycles of 95°C for 30 s, 72°C for 30 s and 72°C for 30 s, with the annealing temperature decreasing 1°C per cycle beginning from 72°C; and an additional 25 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s, with 7 min of final extension at 72°C. PCR products were run in 3% agarose gel and analyzed directly. Samples were sent to COBO Technologies for fragment analysis and Profile IT Solutions (New Delhi, India) indel profiling and quantification.

RESULTS & DISCUSSION

ddPCR is based on mechanically emulsifying a PCR solution into thousands of nanoliter droplets, effectively monitoring thousands of PCR reactions individually and thereby vastly increasing accuracy and reproducibility. It utilizes two fluorescent probes to discern WT and indel sequences: a HEX probe targets the WT gRNA site, and a FAM probe targets a conserved sequence within the amplicon (Figure 1; Supplementary Table 1). Sequences that are WT will give a fluorescent signal for both probes, and sequences possessing indels at the gRNA cut site display only a FAM signal, with the HEX probe failing to anneal. Each PCR-amplified nanoliter droplet is measured for these fluorescent signals, allowing statistically powerful quantification of indels present in PCR reactions. The alternative technique, IDAA, utilizes triple-primer PCR amplification to fluorescently tag the amplicon that includes the gRNA target site (Figure 1) [13]. Amplicons have their base-pair

length measured by capillary gel electrophoresis; the WT length is used as a standard, and sequence lengths that differ are designated indels. The fluorescent signal allows unbiased quantification of amplicons, and the indel size is capable of being determined, importantly, without dependence on prior knowledge of the nature of the indels induced after Cas9:gRNA targeting.

IDAA and ddPCR were tested with a variety of indel mosquito samples to verify their sensitivity toward multiple mutations at the target sites (Figure 1). We examined mosquito samples obtained from a series of small-cage trials of the gene drive AsMCRkh2 strain of the Asian malaria vector mosquito, *A. stephensi* [5,11]. We analyzed 15 pools of 10 mosquitoes each that were considered to have a NHEJ by phenotype selection (white-eye and DsRed-negative) based on previous data [5]. However, because the *kh* mutant white-eye phenotype is associated with a recessive mutation, no phenotypic selection was possible until the second generation (G2). Previous work with these NHEJ mosquitoes had shown that Cas9 indel mutations happened at and around the cut site and protospacer adjacent motif sequence, resulting in insertions and deletions of multiple lengths ranging from 1 to 473 bp [11]. With 185 NHEJ individuals analyzed by Sanger sequencing, 50 different types of indels were identified, including three types of 1-bp indels (from 15 individuals, ~8% of 185) and one type of substitution (from 1 individual, ~0.5%) [11]. Two sets of samples were generated: NHEJ samples, which contained only pools of confirmed NHEJ individuals obtained from previous cage experiments with white-eye and DsRed-negative phenotypes to challenge the sensitivity of the two techniques toward different types of NHEJ, and mixed samples, which were generated by using DNA extracted from a mixture of NHEJ mosquito samples with WT mosquitoes at different proportions to quantify the NHEJ proportion in those samples.

Results from both IDAA and ddPCR experiments for the NHEJ samples showed that both techniques were able to detect all mutant sequences in the NHEJ mosquito samples with an indel percentage of 100%. All samples were analyzed by three technical replicates, with the average total percentage of indels shown in Table 1 and Supplementary Table 2. Both IDAA and ddPCR provide a quantitative analysis of total indel percentage, but only IDAA details the indel sizes and their respective proportions in a sample. Based on the IDAA analysis, indels were detected in a range from 1 to 469 bp, including insertions and deletions, thus representing a broad variety of Cas9-induced indels (Table 2). Many samples contained multiple different indels that were quantified for the proportional amount of each indel present in the sample. Sequencing data for some chosen NHEJ individuals are

Table 2. Insertions or deletion lengths detected by IDAA.

Indel source	Frameshift indels (%)	Length (%)				
		First top indel	Second top indel	Third top indel	Fourth top indel	Fifth top indel
A1-G3	100	2 (52.8)	-2 (47.2)	–	–	–
A1-G8	100	-8 (61.9)	7 (27.0)	-11 (11.1)	–	–
A1-G14	42.8	-11 (34.5)	-3 (27.4)	-6 (20.1)	18 (9.7)	8 (8.3)
A1-G16	100	-13 (85.3)	469 (14.7)	–	–	–
A3-G4	51.2	-48 (48.8)	11 (22.0)	-13 (17.5)	8 (7.3)	-49 (4.5)
A3-G7	100	5 (54.0)	8 (46.0)	–	–	–
A3-G8	100	1 (62.4)	11 (37.6)	–	–	–
A3-G9	53.9	-33 (46.1)	11 (44.7)	1 (4.9)	-34 (4.3)	–
A3-G10	32.1	-33 (65.5)	1 (26.2)	-34 (6.0)	–	–
B1-G4	14.1	-6 (85.9)	-7 (13.0)	-29 (1.1)	–	–
B1-G7	100	-4 (51.5)	1 (27.5)	-14 (11.5)	8 (9.5)	–
B1-G9	100	-4 (54.7)	1 (39.9)	-5 (5.5)	–	–
B1-G10	100	-4 (90.7)	-5 (9.3)	–	–	–
C1-G8	100	-10 (51.6)	2 (20.0)	2 (19.0)	-14 (9.5)	–
C1-G11	100	-4 (90.4)	-5 (9.6)	–	–	–

IDAA allows quantification of each indel sequence of different length, giving insight into the indel composition of the DNA extract. IDAA analysis was done in triplicate (n = 3) with the averages of the top five most prevalent indel lengths displayed from each sample source, the same source used for the analysis detailed in Table 1. Total frameshift indel percentages are provided by excluding indel sizes that are divisible by three. Indels found across all samples range from deletions of 48 bp to insertions as large as 469 bp. Identified mutations included insertions (+) and deletions (-) with different lengths as small as 1 bp. The percentage of each indel is shown in parentheses.
ddPCR: Droplet Digital PCR; IDAA: Indel detection by amplicon analysis; Indel: Insertions or deletion.

listed in Supplementary Table 3, and these confirmed the sensitivity accuracy of IDAA and ddPCR for different types of indels. Not all indels were identified with Sanger sequencing because of the time and labor costs necessary to extract and sequence all individuals, limited sources of genomic DNA from single mosquito extractions and PCR technical problems. In addition, not all samples were suitable for Sanger sequencing because DNA was extracted from mosquito pools and included a mixture of mutations. This level of complexity reduced the reliability of PCR amplification because not all of the mutations could be amplified with the same efficiency due to variants of different indel frequencies, resulting in nonspecific sequencing errors. Providing sequencing data for each sample via next-generation sequencing would costly, unnecessary and difficult due to the abovementioned reasons regarding the quality of DNA extracts and amplification of different indels in a pooled sample.

Sensitivity and quantification of 1-bp insertions by IDAA can be seen in samples G8A3, G9A3, G10A3, G7B1 and G9B1 (Table 2 & Supplementary Table 3). The same DNA extracts from all samples were used for both techniques, allowing a direct comparison of indel quantification. Because the ddPCR technique designated the same sample extracts at or near 100% indel, it demonstrates that the 1-bp insertions in those samples are being reliably detected (Table 1). This is consistent with prior data supporting the 1-bp indel sensitivity of both IDAA and ddPCR [13,14]. Overall, every indel size discovered by the IDAA method was detected by ddPCR, as it designated all samples as 100% or near 100% indel with no significant differences observed between individual samples and a strong correlation coefficient of 0.73 (Table 1). If ddPCR were insensitive to a certain indel identified in a sample by IDAA, then the total indel percent determined by ddPCR would proportionally reflect an increase in WT percentage. Samples G8A1, G9A3, G7B1, G10B1 and G11C1 were slightly below 100% indel frequency in either technique, and this is likely due to fluorescence anomalies. The nominal WT sequence quantified in these samples (0.03–0.8%) is unreliable because, at its lowest frequency, a true WT allele in a pool of ten indel mosquitoes (20 alleles total) would produce a 5% WT (or 95% indel) proportion, which was not observed.

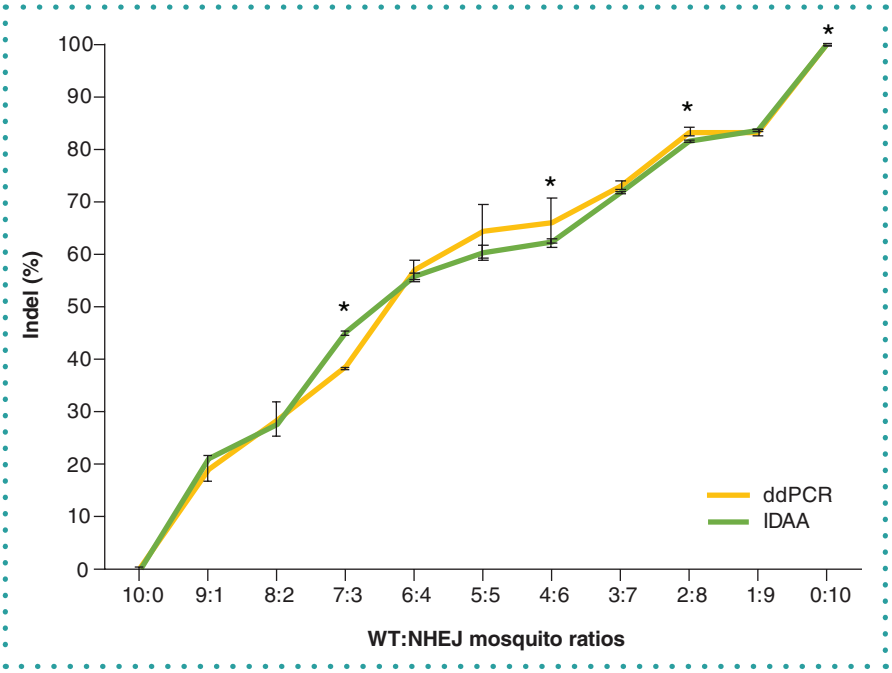

Figure 2. Quantification of nonhomologous end joining alleles in AsMCRkh2 mosquito samples by ddPCR and IDAA techniques. DNA was extracted from 15 to 10 mosquito pools. To assess the sensitivity of both techniques, the mosquito pools consisted of a mix of WT and NHEJ mosquitoes at 11 different ratios of WT:NHEJ (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10). DNA was used for PCR, and amplicons were subjected to IDAA and ddPCR analysis to determine the indel percentage in each sample. Each ratio was conducted in triplicate (n = 3), and average results were compared between the two techniques. Although deviating from theoretical indel percentages (40% indel for a 6:4 ratio), both techniques demonstrated precision based on producing similar results for each ratio and having a Pearson correlation coefficient of r = 0.99. In addition, values provided by ddPCR and IDAA are also representative of their accuracy; because both deduced the same indel percentage, it is likely close to the actual indel percentage. Student's t test was performed to compare the measurements at each ratio (*p < 0.05).
ddPCR: Droplet Digital PCR; IDAA: Indel detection by amplicon analysis; Indel: Insertions or deletion; NHEJ: Nonhomologous end joining; WT: Wild-type.

In order to assess accuracy and replicate a trial scenario in which quantification techniques are employed, 11 pooled samples of NHEJ mosquitoes were made with WT mosquitoes at different ratios of WT:NHEJ mosquitoes (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10) within a pool of 10 total mosquitoes (Figure 2 & Table 2). In the mixture of NHEJ mosquitoes from the A3 to G4A3-G4 samples with multiple types of mutations, both ddPCR and IDAA techniques showed indel frequencies similar to theoretical frequencies (e.g., 5:5 ratio sample produces 50% indel and 50% WT), as well as both techniques having similar percentages, and no statistical differences were observed for the majority of samples. Also, a similar trend in the deviation from theoretical frequencies can be observed in both techniques as supported by a correlation coefficient of 0.99 (Figure 2). Statistically significant differences were observed for the 7:3, 4:6, 2:8 and 10:0 ratios between the IDAA and ddPCR percentages, which may be due to the detection or binding of fluorescent probes/primers and unequal amplification during PCR processes. Moreover, IDAA allows the identification of indel sizes, enabling the approach of tracking an indel as it is inherited through multiple generations as previously shown when indel germline transmission rates were traced in zebrafish [15]. Samples from different mosquito generations of the same cage population can be used to identify multiple indels across several generations (-4, 1 and -5) (Figure 3A). Randomly chosen individuals analyzed with Sanger sequencing confirmed the results obtained by IDAA and ddPCR and showed that the detected indels are accurate (Figure 3B). Some indels were identified by IDAA but were not identified with Sanger sequencing (-16 in G7 and +1 in

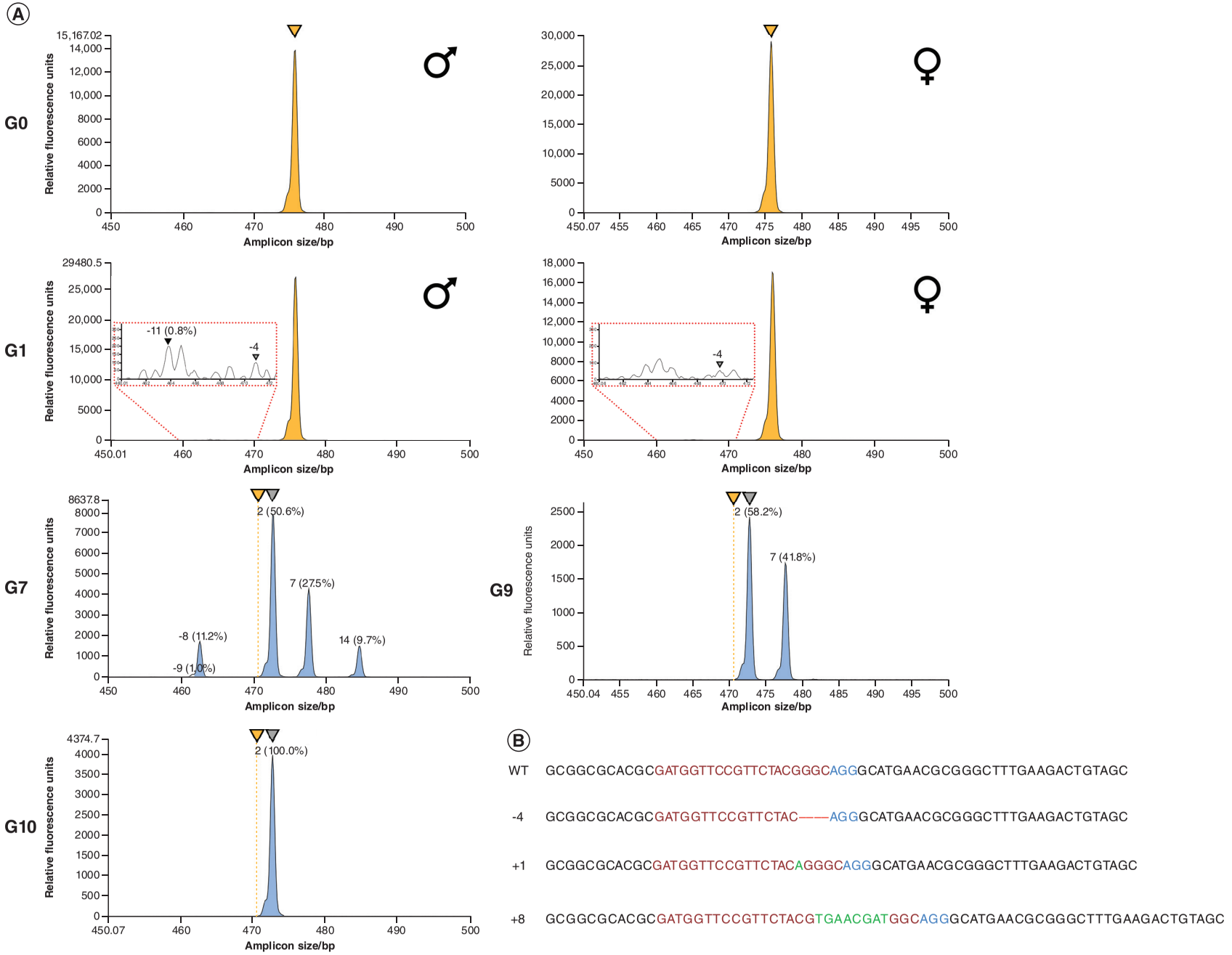
A
G0
♂
Relative fluorescence units
15,167.02
14,000
12,000
10,000
8000
6000
4000
2000
0
450
460
470
480
490
500
Amplicon size/bp
♀
30,000
25,000
20,000
15,000
10,000
5000
0
450.07
455
460
465
470
475
480
485
490
495
500
G1
29480.5
25,000
20,000
15,000
10,000
5000
0
-11 (0.8%)
-4
450.01
18,000
16,000
14,000
12,000
10,000
8000
6000
4000
2000
G7
8637.8
8000
7000
6000
5000
4000
3000
2000
1000
2 (50.6%)
7 (27.5%)
-8 (11.2%)
14 (9.7%)
-9 (1.0%)
G9
2500
2000
1500
1000
500
2 (58.2%)
7 (41.8%)
G10
4374.7
4000
3500
3000
2500
2000
1500
1000
500
2 (100.0%)
450.07
450.04
B
WT GCGGCGCACGCGATGGTTCCGTTCTACGGGCAGGGCATGAACGCGGGCTTTGAAGACTGTAGC
-4 GCGGCGCACGCGATGGTTCCGTTCTAC——AGGGCATGAACGCGGGCTTTGAAGACTGTAGC
+1 GCGGCGCACGCGATGGTTCCGTTCTACAGGGCAGGGCATGAACGCGGGCTTTGAAGACTGTAGC
+8 GCGGCGCACGCGATGGTTCCGTTCTACGTGAACGATGGCAGGGCATGAACGCGGGCTTTGAAGACTGTAGC

Figure 3. Tracing and quantification of insertions or deletions in AsMCRkh2 mosquito samples over generations (see facing page). G0 and G1 mosquito samples were chosen randomly, and samples from G7 through G10 were all individuals carrying NHEJ alleles selected by phenotype (white-eyed/DsRed-negative). (A) G0: 'Founder' individuals show the baseline for the IDAA profile. Both males and females present a WT sequence at the target site shown by a yellow peak (because all female G0s are WT, whereas even though G0 males were a combination of transgenic and WT males only WT [and NHEJ alleles, if there were any] were amplified by PCR). G1: First-generation offspring display expected low frequency of indels in sample pools of WT and low-frequency NHEJ individuals. Red-dotted line zoom-in inserts display the rarely occurring NHEJ indel events in the population (<0.8%), and the black and gray triangles indicate spectra peaks of indels. Two types of indels, -11 and -4, were identified in G1. IDAA and ddPCR allowed the analysis of a large number of samples from G0 and G1, which was required to determine NHEJ allele-generated frequency. G7–G10: White-eyed phenotype mosquitoes with homozygous NHEJ alleles were selected from different generations. Although phenotypically similar, the variable peak heights indicate that G7 individuals represent a heterogeneous population, with different types of indels, whereas G9 (near equal peak heights) and G10 (single peak) represent a homogeneous population with only one indel (-4) selectively carried to subsequence generations. WT alleles are distinguished by yellow peaks when present in the spectra; when absent, yellow triangles above the spectra panels are used to reference the WT location. Frameshift-causing indels are indicated with peaks color-coded in blue. (B) Sanger sequencing in mosquitoes from G7 (11 individuals), G9 (5 individuals) and G10 (16 individuals) show results comparable with the IDAA findings. Three types of indels (-4, +1, +8) were identified in G7 mosquitoes, whereas only one type of indel (-4) was present in G9 and G10 (Supplementary Table 3).
ddPCR: Droplet Digital PCR; IDAA: Indel detection by amplicon analysis; Indel: Insertions or deletion; NHEJ: Nonhomologous end joining; WT: Wild-type.

G9), indicating that IDAA allows a broader coverage of analysis and prevents missing important indels due to small samples size as when analyzed by Sanger sequencing.

Both IDAA and ddPCR have beneficial characteristics beyond their technical capabilities, including the cost and timeline for acquiring large datasets. The exact cost of these techniques for a project is difficult to compare because the prices for services vary among institutions and depending on where the techniques are sourced. An estimation of the total cost per sample for either ddPCR or IDAA is around $20. Reagent costs vary based on the amount purchased, but become negligible compared with analysis costs in a large experiment. For our purposes, both IDAA and ddPCR had comparable costs of reagents, with the latter having lower operational costs due to being performed at a nonprofit UCI facility. If instrumentation is at hand, the workbench procedure for IDAA, PCR amplification using the triple-primer PCR protocol, can be completed within a day [16]. Subsequently, the samples can be shipped to COBO Technologies for analysis, and the results can be obtained in less than a week, or within days in 'fast track' mode, after samples are received. Samples for ddPCR can be fully prepped, assayed and analyzed in a single day if instrumentation is available.

Both methods are far more cost effective than deep-sequencing techniques. For example, the Illumina MiSeq™ System (CA, USA) platform price is approximately $400 to run a variable number of reactions and an additional $95 per reaction for library preparation. Moreover, analysis of deep sequencing data requires significant bioinformatics expertise, which is not required for either IDAA or ddPCR. High-resolution melting analysis is another cost-effective, viable option for mutation analysis and genotyping but lacks the quantification capabilities of ddPCR and IDAA. The detectable threshold is higher for high-resolution melting analysis at 10%, whereas IDAA and ddPCR are more sensitive, detecting mutant sequences as low as 0.1% [17]. Although IDAA is capable of providing more information on indel sizes and relative composition, most of the indels are observed within ±20 bp from the putative double strand DNA break site in another mosquito species (*Aedes aegypti*); therefore, ddPCR and IDAA cannot detect any large deletions beyond the window of ±20 bp [18]. Assay wipeout can occur for ddPCR if a deletion is large enough to disrupt the sequence of the reference probe (FAM) binding site; in this case, no probes are bound, resulting in a false-negative signal. Guidelines specify that the reference probe should be at least 25 bps from the gRNA cut site to prevent this, so, depending on the distance used in assay design, the potential for this occurrence is variable.

In these comparative experiments, we demonstrated that ddPCR and IDAA are promising techniques for the quantification and analysis of NHEJ alleles in gene drive mosquitoes. These techniques showed sensitive and reproducible detection of NHEJ events and can be used instead of next-generation sequencing for a high-throughput protocol that saves time and reduces cost. This approach offers a more efficient analysis of gene-drive cage experiments and field trials where quantification of NHEJ is important as an indicator of potential resistance alleles that can prevent complete drive introduction into field populations [11,19]. Both techniques have their strengths and weakness depending on the purposes of the user. Because IDAA detects indels uniquely by length deviations, the technique will overlook point mutations such as substitutions, which were rare events in mosquito gene-drive systems [4,5,11]. In addition, highly variable or nonconserved DNA regions may not be suitable for IDAA analysis because of the presence of preoccurring indels that interfere with the detection of NHEJ-induced indels. Because IDAA cannot detect SNPs and the current application is to quantify NHEJ-induced indels, the analysis of SNPs in this experiment was omitted, although ddPCR had been shown to detect SNPs as mutant sequences [20]. However, IDAA provides the percentage of each different indel in a mixture, and this information can be useful for tracking mutations through successive generations in a cage trial format or in open release trials as a surveillance approach. In contrast, ddPCR identifies mutations by binding of probes at the target site and thus has greater sensitivity for all types of indels. The detection of SNPs could potentially interfere with a NHEJ quantification assay, due to the same observable output between SNPs and indels (failure of gRNA cut site probe to anneal). A benefit of ddPCR is that the equipment can be easily transported, which is suitable for analyzing gene drive efficiency in the field where resources for sample prep, shipment and analysis are limited. Unlike IDAA, ddPCR can detect substitution mutations but is not effective for tracking indels over generations. Potentially the largest drawback shared by both techniques is the lack of sequence data, given such information is pertinent for answering the research question. In the case of a screening application where

samples with indels are rare, ddPCR or IDAA can be coupled with sequencing to acquire sequence data while maintaining high-throughput efficiency.

IDAA and ddPCR showed sensitive and reproducible detection of NHEJ events in mosquito samples from cage experiments. Both techniques offer a more efficient analysis of indel quantification in a cost- and time-saving manner, and they can be used for efficient analysis of gene-drive mosquito populations for quantifying NHEJ. Thus, they possess the qualifications to determine factors that will influence gene drive in cage trials or field releases.

FUTURE PERSPECTIVE

As CRISPR-Cas9-based gene drives are being widely developed for applications in vector control, ecology conservation and pest management, cage trials and field trials will likely become regulatory checkpoints for deploying gene drives in living organisms into the field. A high-throughput yet cost-effective method to determine NHEJ alleles compared with HDR for drive efficiency is necessary for the study of gene-drive behaviors in big population samples format.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at www.future-science.com/doi/suppl/10.2144/btn-2019-0103

AUTHOR CONTRIBUTIONS

R Carballar-Lejarazú: Study conception and design, data analysis and interpretation, manuscript drafting and revision, approval for publishing and agreement to be accountable for the study and manuscript. A Kelsey: Study design and investigation, data collection, analysis and interpretation; manuscript drafting and revision; approval for publishing; and agreement to be accountable for the study and manuscript. TB Pham: Study design and investigation; data collection, analysis and interpretation; manuscript drafting and revision; approval for publishing; and agreement to be accountable for the study and manuscript. EP Bennett: IDAA indel profiling and quantification, and manuscript revision. AA James: Data interpretation and manuscript revision.

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No writing assistance was utilized in the production of this manuscript.

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BioTechniques

CRISPR VS COVID-19: HOW CAN GENE EDITING HELP BEAT A VIRUS?

Known to be a sturdy weapon in a scientist's arsenal, how has the gene editing tool CRISPR been applied in the fight against COVID-19?

When SARS-CoV-2 was first identified, many researchers redirected their focus to the study of this novel virus and the disease it causes. Those working with CRISPR were no exception, and the gene editing tool was soon brought to the frontlines in the worldwide war against COVID-19. With the technology based upon a naturally occurring bacterial gene editing system that plays a key role the prokaryotic defense against viral infection, the CRISPR—Cas system is designed to fight viruses. The challenge facing researchers now is how best to utilize its natural ability and optimize it for human benefit.

Here, scientists have indeed risen to the occasion and CRISPR technology has been successfully used to develop rapid diagnostic tests for COVID-19 – gaining its first US FDA (MD, USA) approval in the process [1]. Research continues as such tests are now being developed for widespread clinical use, with multiple companies in the race to fill the ever-widening gap in the market left by reagents for PCR-based COVID-19 tests running out and dwindling testing capacity. In other areas, scientists have looked to CRISPR as a potential therapeutic, utilizing its targeted enzymatic activity to destroy SARS-CoV-2 RNA and prevent viral replication.

CRISPR has been well and truly deployed in the fight against COVID-19, but what potential does it hold in controlling this and future pandemics?

TARGETED DETECTION WITH CRISPR-BASED DIAGNOSTICS

Testing has been a key factor in many nations' COVID-19 response policies, with PCR-based testing taking center stage as the gold standard of diagnostic tests. However, a long wait for results, a labor-intensive protocol and a dwindling supply of reagents has led to many looking for alternative testing options. To keep up with the mass testing protocols that many experts have deemed necessary to control the spread of the virus, the development of a rapid, at-home diagnostic test could help to turn the tide on the ever-increasing global case numbers. For this, many have turned to CRISPR, with its nucleotide-targeting ability making it optimal for detecting the presence of viral RNA.

Utilizing CRISPR as a diagnostic tool is not a novel idea; the companies Sherlock Biosciences (MA, USA) and Mammoth Biosciences (CA, USA) – co-founded by CRISPR pioneers Feng Zhang and Jennifer Doudna, respectively – were each launched with the goal of developing CRISPR-based diagnostic tools [2,3]. At the start of the COVID-19 pandemic, both companies shifted their attention towards creating a system that could detect the SARS-CoV-2 virus, and in May 2020 it was Sherlock Biosciences that made history in gaining the first FDA-authorized use of CRISPR. ▶

Speaking to *BioTechniques*, Sherlock co-founder and CEO Rahul Dhanda noted, "*We at Sherlock see this as a historic moment for the organization but also for the field in general because what has always been this very robust technology in CRISPR has become validated as a real solution to healthcare*."

Their SHERLOCK™ CRISPR SARS-CoV-2 test kit was granted Emergency Use Authorization from the federal authority, meaning it can be used to test for SARS-CoV-2 in certified laboratories. The COVID-19 test kit works by programming the CRISPR system to detect the genetic signature of SARS-CoV-2, utilizing gRNA that is complementary to a specific section of the viral genome. If the target section is detected in a sample, the CRISPR system is activated and a detectable signal – in this case a fluorescent marker – is released [4,5].

"*So far the accuracy has been incredibly high*," explained Dhanda when asked about the benefits of SHERLOCK relative to PCR-based testing. "*We have done our own critical analysis for the FDA authorization that demonstrated 100% accuracy, sensitivity and specificity. In addition, it is much faster; while PCR can take a number of hours, this is done in about an hour. The other benefit is it is relying on another set of reagents and so some of the limitations in the supply chain that have been affecting the use of RT-PCR are not necessarily limiting Sherlock's test*."

While the technology is promising, CRISPR-based tests are still limited to the lab. The real challenge now is in creating a point-of-care testing system that enables rapid, accurate testing in any setting. To achieve this, Sherlock are in collaboration with multiple partners, for example, binx health (MA, USA) with whom they are working alongside to create the world's first point-of-care diagnostic test for COVID-19. Already this collaboration has brought the turnaround time of the test down to 20 minutes, and it is hoped that the large-scale production of such a test will bring the price per test down and increase accessibility.

CRISPR-BASED COMPETITION: CREATING THE FIRST CLINICALLY VIABLE TEST

While Sherlock made history in gaining FDA approval for their test, developing a clinically viable counterpart is still hotly contested. The race to create a viable CRISPR-based diagnostic is close, and the DETECTR™ assay from a group affiliated with Mammoth Biosciences provides a second option for an alternative COVID-19 test. With a CRISPR–Cas12-based system, the DETECTR test is able to identify the presence of the virus from nasopharyngeal or oropharyngeal swab samples [6]. As with the SHERLOCK system, the test has a quick turnaround time, does not require the complex lab equipment and has displayed similar levels of sensitivity and specificity to qRT-PCR tests.

"*We need faster, more accessible and scalable diagnostics*," commented Mammoth CTO Janice Chen. "*The point-of-care testing space is ripe for disruption and CRISPR diagnostics have the potential to bring reliable testing to the most vulnerable environments*" [7].

Outside of the biotech industry sector, academic research groups are also throwing their hat into the ring to create a test. Publishing their work in *PLoS Pathogens*, a Chinese research group recently announced the development of their CRISPR-COVID test [8]. Described by the authors as "*an isothermal, CRISPR-based diagnostic for COVID-19 with near single-copy sensitivity*," their test takes just 40 minutes to produce results.

In addition, recently researchers from the University of Connecticut (USA) validated the clinical feasibility of their All-In-One-Dual CRISPR-Cas12a (AIOD-CRISPR) method, utilizing a low-cost hand warmer as an incubator and generating results in as little as 20 minutes. By using a hand warmer, the researchers eliminated the need for an electrical incubator and thus created an instrument-free point-of-care diagnostic [9]. "*Such a simple, portable and sensitive detection platform has the potential to provide rapid and early diagnostics of COVID-19 and other infectious diseases at home, in the doctor's office, and even at drive-thru testing sites*," commented group leader Changchun Liu [10].

With multiple groups working towards the goal of a CRISPR-based diagnostic, it is just a matter of time before the technique is cleared for clinical use. In the timeline of a pandemic, this cannot come soon enough, with many having already highlighted the need for mass testing with cheap, rapid point-of-care tests in order to overcome a deadly second wave [11]. However, whether ready in time to have clinical benefit in this pandemic or in the next, precedent has been set for CRISPR as a diagnostic and once again it has proven itself a vital lab method in creating the next generation of tests.

PAC-MAN VS THE PANDEMIC: THERAPEUTICS GOING RETRO

Therapeutic applications for CRISPR are on the rise, with the technology playing a key role in the development of potential cures for a variety of genetic diseases by directly editing the genome [12]. Meanwhile, taking a different approach and looking outside the human genome, researchers from Stanford University (CA, USA) are working towards a CRISPR-based therapeutic for infectious disease. Beginning their work targeting the influenza virus, the team have followed suit of many before them and refocused the aim of their gene-targeting antiviral agent towards COVID-19 and the global battle against the pandemic [13].

The Prophylactic Antiviral CRISPR in huMAN cells – or PAC-MAN – technology includes the Cas13 enzyme and a strand of gRNA that is specific to nucleotide sequences in the SARS-CoV-2 genome. In targeting and subsequently destroying the viral genome, PAC-MAN technology effectively eliminates the threat of the virus by preventing viral replication. Further, by targeting RNA sequences that are conserved across all members of the *Coronaviridae* family, the researchers suggest that PAC-MAN could become a pan-coronavirus inhibition strategy that is effective against all disease-causing coronaviruses [13].

While PAC-MAN technology has already proven its worth as a molecular tool, translating this into a clinically viable treatment has its issues – the most prominent being the lack of an effective delivery mechanism. This is a fundamental challenge faced across the field of gene editing, as the component parts of CRISPR are just too large to enter the target cells [14]. Various delivery mechanisms are being investigated to overcome this issue, and the Stanford team have collaborated with a group at the Molecular Foundry (CA, USA) who specialize in the development of synthetic molecules called lipitoids which hold potential as an effective CRISPR delivery system.

Lipitoids – a type of synthetic peptide – have been shown to be nontoxic to humans and are able to effectively deliver nucleotides to cells by encapsulating them into nanoparticles approximately the size of a virus. When combined with the PAC-MAN technology, the lipitoid Lipitoid 1 performed well and, in a sample of human epithelial lung cells, was effective at reducing the amount of SARS-CoV-2 in a solution by over 90% [15]. The next step for the Stanford-based group is to test their PAC-MAN/lipitoid therapy in an animal model against a live SARS-CoV-2 virus.

"An effective lipitoid delivery, coupled with CRISPR targeting, could enable a very powerful strategy for fighting viral disease not only against COVID-19 but possibly against newly viral strains with pandemic potential," commented Michael Connolly, leader of the Molecular Foundry group [15].

LOSE THE BATTLE, WIN THE WAR

Even in the fast-paced field of COVID-19 research, a clinically viable CRISPR-based therapeutic may be a long way off. While COVID-19 may beat CRISPR in this current battle, the pan-coronavirus applications of the PAC-MAN technology likely mean that CRISPR will come out on top in the war against future coronaviruses.

In the current COVID-19-centric news cycle, advances not directly related to COVID-19 treatments are often viewed as a side note. Attention is thoroughly focused on what developments can do to rid the world of the ongoing pandemic and get things 'back to normal'. That being said, it is worth noting that the technology developed in the face of COVID-19 can be applicable for many other infectious diseases, and the advances in CRISPR technology developed today will likely be beneficial for decades to come.

Written by Jenny Straiton

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Expert Opinion

BioTechniques

Empowering chimeric antigen receptor T-cell therapy with CRISPR

Xuanzhu Zhou*[1]

Ever since the first chimeric antigen receptor T- (CAR-T) cell therapy, Kite Pharma's Yescarta® for treating non-Hodgkin lymphoma, was approved by the US FDA in October 2017, numerous CAR-T-cell therapies for a variety of cancer types have been granted Investigational New Drug clearance and entered clinical phases. CAR-T therapy researchers are now fueled with enthusiasm and optimism, aiming to declare cancer a curable disease.

WHAT IS CAR-T-CELL THERAPY?

CAR-T-cell therapy involves using engineered T cells expressing tumor antigen-specific CARs for targeting cancer cells. A basic CAR consists of three parts: one extracellular antigen recognition domain, one transmembrane domain and one intracellular signaling domain. The antigen recognition domain is typically a single-chain variable fragment derived from a monoclonal antibody against tumor-associated cell surface antigens. The transmembrane domain of a CAR is commonly from CD28, which provides stability to the CAR. The intracellular signaling domain is generally comprised of a CD3ζ structure, costimulatory molecules and/or cytokine expression cassettes, for enhanced downstream signaling and T-cell function [1].

> ***"Exploration of other potential CRISPR/Cas9 gene targets for multiplex editing is of greatest interest now for developing the optimal 'off-the-shelf' allogeneic CAR-T cells."***

KEYWORDS

CAR-T cell therapy • CRISPR • CRISPR HDR template

[1]GenScript USA Inc., Piscataway, 08854 NJ, USA; *Author for correspondence: claire.zhou@genscript.com

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AUTOLOGOUS VERSUS ALLOGENEIC CAR-T THERAPY

Depending on the source of the T cells, CAR-T therapy can be classified into two categories: autologous or allogeneic. In autologous CAR-T-cell therapy, T cells derived from a patient are engineered as 'living drugs' to recognize and attack the patient's own cancer cells. This patient-specific treatment involves the collection, preservation, shipment, genetic engineering and readministration of T cells from and into the same patient [2]. Currently, the two CAR-T-cell therapies approved by the FDA (Yescarta and Novartis' Kymriah®) are both autologous therapies. Despite the promising results shown in treating certain forms of hematopoietic malignancies, autologous therapy also has its limitations. Not only is it time consuming (which can be extremely critical for late-stage cancer patients), but it also comes with astonishing price tags (US$373,000 for Yescarta and US$475,000 for Kymriah) [3]. In addition, it is not always possible to collect and manufacture enough functional T cells from patients who may already be lymphopenic due to previous treatments or their disease status.

To overcome the limitations of autologous CAR-T-cell therapy, some researchers have moved on to developing allogeneic CAR-T cell therapy, which involves engineering T cells isolated from healthy donors to target patients' tumor-associated antigens in order to defeat cancer. This allogeneic approach is able to bypass the limitations of autologous cell therapy, as T cells from healthy donors are easier to collect and can be engineered with a more controlled and streamlined manufacturing process. However, it poses several other risks to the patients. Because the allogeneic T cells have been isolated from foreign donors, patients may face graft-versus-host disease resulting from human leukocyte antigen mismatch mediated by the donor's T-cell αβ receptor (TCR-αβ) and/or host-versus-graft treatment rejection due to the patient's T-cell human leukocyte antigen mismatch [4]. The most promising strategy to date to circumvent these issues is to couple the power of genome editing, especially the revolutionary CRISPR/Cas9 genome editing technology, with CAR-T engineering.

HOW IS CRISPR/CAS9 USED IN DEVELOPING ALLOGENEIC CAR-T THERAPY?

One essential decision to make in designing CAR-T cells is to choose the correct DNA template for CAR expression. An ideal DNA template has the following qualities: allows flexible insert sizes, inserts at target sites with high efficiency, is highly specific with no off-target insertion, has low cellular toxicity and can be obtained easily and rapidly. Traditionally, viral vectors are used for the delivery of CARs due to their high transduction efficiency. The most commonly used viral vector systems are γ-retroviruses, lentiviruses, adenoviruses and adeno-associated viruses [5]. However, when using viral vectors, there are always concerns regarding their integration into the wrong genome location, which may lead to the onset of other diseases [6]. With the maturation of CRISPR/Cas9 technology, it is now possible to insert large genes at specific genetic sites in T cells for CAR-T engineering without using

viral vectors, minimizing off-target integrations [7].

In 2013, CRISPR/Cas9 technology was first described as a powerful eukaryotic cell genome editing tool, which can create precise double-stranded breaks (DSBs) at a predefined target DNA site and lead to gene mutation. Now, it is widely used in both research and clinical studies. There are two essential components of CRISPR technology: a guide RNA (gRNA) designed for recognizing the protospacer adjacent motif sequence on target DNA, and a Cas9 protein that exerts endonuclease function for creating DSBs. The DSBs will trigger two distinct mechanisms for repair. One mechanism is through the nonhomologous end joining (NHEJ) repair pathway, which introduces mutations to the DSB sites, leading to gene knockout. The other DSB repair mechanism is homology-directed repair (HDR), which enables the donor DNA templates to be accurately inserted at the break sites for gene knock-in [8]. By employing CRISPR HDR pathway, researchers are able to precisely insert a CAR expression cassette into T cells without using viral vectors.

MULTIPLEX EDITING WITH CRISPR/CAS9 FOR PREVENTING ALLOGENEIC CAR-T SIDE EFFECTS

Taking advantage of the multiplex gene editing capability of CRISPR/Cas9, potential safety issues associated with allogeneic CAR-T therapy can be addressed simultaneously during CAR insertion. For example, to prevent graft-versus-host disease, the general approach is to knockout the expression of TCR-αβ of the donor T cells. TCR-αβ heterodimer function requires the expression of both α- and β-chains [9]. In order to disrupt TCR-αβ donor T-cell expression, the α-chain can be knocked out by using CRISPR gRNA specific to the gene encoding TCR-α, *TRAC*. Eyquem *et al.* showed that, by targeting CAR insertion to the *TRAC* exon, CAR expression can be placed under the control of endogenous transcriptional regulation, leading to sustained T-cell function and delayed cell exhaustion [10]. Host-versus-graft rejection can also be resolved by knocking out β_2-microglobulin, part of major histocompatibility complex class I molecules, using CRISPR to prevent surface alloantigen presentation [11]. Moreover, multiplexing additional gRNAs targeting immune inhibitory receptors, such as PD-1 or LAG-3, has shown potential benefits in enhancing the antitumor activity of CAR-T cells [12–14]. Exploration of other potential CRISPR/Cas9 gene targets for multiplex editing is of greatest interest now for developing the optimal 'off-the-shelf' allogeneic CAR-T cells.

KEY CONSIDERATIONS FOR EMPLOYING CRISPR/CAS9 HDR PATHWAY FOR T-CELL ENGINEERING

CRISPR/Cas9 delivery format: use ribonucleoprotein system for optimal delivery

To eliminate the chance of introducing any foreign DNA and inducing insertional mutagenesis, it is best practice to deliver the CRISPR/Cas9 system in the form of ribonucleoprotein, a complex formed by incubating Cas9 protein and gRNA together [15]. Purified Cas9 proteins, as well as other Cas proteins (e.g., Cpf1) are readily available from different vendors. Chemically synthesized full-length gRNA (about 100 nucleotides long) is preferred over *in vitro* transcribed gRNA, which has been shown to induce strong innate immune responses. Another important factor to consider when choosing a gRNA provider is its purification method. High-purity synthetic gRNA purified via HPLC is recommended, with a higher ratio of full-length gRNA products and homogeneity leading to a higher editing efficiency and consistency.

HDR DNA donor template format: ssDNA versus dsDNA

The major concern with regard to gene-edited CAR-T cells is off-target integration [16], which is predominantly influenced by the HDR donor template format. The HDR DNA donor template requires one homology arm (typically around 500 bp) on each side of the gene insert flanking the DNA cut site. Traditionally, dsDNA was widely used due to its ease of production via PCR amplification, whereas producing long ssDNA was almost impossible. However, dsDNA donors are associated with high off-target integration rates, as they can be inserted via not only the HDR pathway but also the error-prone NHEJ process. Incorporation through NHEJ could result in duplication of the homology arms at gRNA target sites, integration at off-target sites or even random insertion to endogenous DSBs that have occurred naturally [17–19]. When compared with dsDNA, ssDNA demonstrates significantly improved editing specificity and reduces off-target integration. A recent study published by Roth *et al.* in *Nature* demonstrated that ssDNA templates have similar gene knock-in efficiency but significantly reduced off-target integration (by over 20-fold) compared with dsDNAs in T cells [20]. These characteristics make ssDNA the ideal template for CRISPR-based gene insertion and replacement. Recent technology breakthroughs have made long ssDNA encoding large genes, up to 3 kb or longer, commercially available to expedite the development of safer CAR-T cells.

Other factors to consider may include the possibility of increasing HDR efficiency, as HDR predominantly occurs at the S/G2 phase of the cell cycle. Several methods have been developed to upregulate HDR, including suppressing NHEJ and activating HDR using chemical and genetic approaches, cell cycle manipulation to prolong the S/G2 phase and colocalization of CRIPSR ribonucleoprotein and ssDNA donor to targeted DSBs via covalent bonding.

FUTURE PERSPECTIVE

From autologous to allogeneic CAR-T cells, from treating liquid tumors to solid tumors, the development of CAR-T-cell therapy is marking a new era in immunotherapy. Using a CRISPR/Cas9-mediated, locus-specific HDR pathway for CAR insertion and ssDNA as an HDR template in T-cell engineering holds great promises in developing safer and more effective 'off-the-shelf' CAR-T-cell products as a universal treatment solution.

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Reports

Cellular thermal shift analysis for interrogation of CRISPR-assisted proteomic changes

Nam-Gu Her[1,2], Ivan Babic[3], Venkata M Yenugonda[3], Santosh Kesari[3] & Elmar Nurmemmedov*[,3]

ABSTRACT

CRISPR–Cas9 has proven to be a versatile tool for the discovery of essential genetic elements involved in various disease states. CRISPR-assisted dense mutagenesis focused on therapeutically challenging protein complexes allows us to systematically perturb protein-coding sequences *in situ* and correlate them with functional readouts. Such perturbations can mimic targeting by therapeutics and serve as a foundation for the discovery of highly specific modulators. However, translation of such genomics data has been challenging due to the missing link for proteomics under the physiological state of the cell. We present a method based on cellular thermal shift assays to easily interrogate proteomic shifts generated by CRISPR-assisted dense mutagenesis, as well as a case focused on NuRD epigenetic complex.

METHOD SUMMARY

In this study, we used the CRISPR–Cas9 tool to introduce mutations (3–9 bp) to several components of the NuRD complex in HUDEP-2 cells. We then generated stable HUDEP-2 clones harboring these mutations. Using the cellular thermal shift assay method, we quantified the abundance of each of mutated proteins in every clone and thus were able to analyze the effect of these proteomic perturbations on the stability of the NuRD complex.

KEYWORDS

cellular thermal shift • CETSA • CRISPR • protein complex

*[1]Korea Institute of Radiological & Medical Sciences, Nowon, Seoul 01812, Korea; [2]Samsung Medical Center, Gangnam, Seoul 06351, Korea; [3]John Wayne Cancer Institute & Pacific Neuroscience Institute, Providence Saint John's Health Center, Santa Monica, CA 90404, USA; *Author for correspondence: elmar.nurmammadov@providence.org*

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CRISPR, in combination with Cas9 nuclease, can serve as a robust technology for deciphering the functional consequences of gene editing. With its number of applications expanding, the CRISPR–Cas9 system is the most promising and broadly developed tool for targeted genome engineering [1,2]. When guided Cas9 cleaves a gene locus, imprecise end joining repair of the cell is activated, most often leading to disrupted gene function. This paradigm allows for the systematic dissection of genetic dependencies in genome-wide CRISPR screens in the context of disease-relevant cellular phenotypes [1,3–5]. CRISPR tiling, a more recent application of this technology, offers comprehensive dense mutagenesis with many cleavages per gene to systematically define functional protein-coding sequences [6,7]. Following such screens, protein and nucleotide sequence level annotations, as well as 3D visualization of protein structures, can be performed to gain mechanistic insight and to elucidate functional residues important to the stability of proteins [8,9]. These analyses identify discrete protein sequences that are essential for specific biological phenotypes. The mechanisms by which individual protein elements contribute to various cellular states could aid in the rational design of novel therapeutics and guide biological engineering.

Translation of data from CRISPR tiling screens into an evaluation of protein stability affected by introduced mutations is a challenging task. The cellular thermal shift assay (CETSA) has emerged as a method that allows quantitative measurement of the stability of individual proteins within the physiological context of a cell. CETSA is typically used as a method to evaluate thermal stabilization of a protein bound to a ligand. The assay relies on the strength of the interaction of a given ligand with the target protein [10,11]. The perturbations caused by the binding of small molecules cause thermodynamic shifts, which in turn are reflected in the thermal stability and free energy state of the protein [12–14]. The versatility of this method allows its use with any cell type when the investigated protein is in its physiological environment, a key advantage of combining CETSA with CRISPR [15,16]. Point mutations introduced into protein-coding regions of genes can similarly cause thermodynamic shifts that will destabilize the individual target protein and its ensuing protein complex. CRISPR tiling mimics drug screening, where each point mutation represents a perturbation caused by a small molecule. Taken together, CRISPR tiling can identify protein regions where targeting with small molecules can yield maximum therapeutic effect without causing unnecessary toxicity. We present a CETSA-based method to easily investigate proteomic shifts generated by CRISPR-assisted dense mutagenesis.

The NuRD repressive complex was chosen as our model for demonstration purposes, as it represents an architecturally challenging, multifunctional and yet therapeutically important protein complex. The NuRD complex emerges in the context of therapeutic targets that have the potential to reprogram cells and thereby promote tissue regeneration and repair and reverse the oncogenic activity of cancer cells [17]. Although initially identified as a transcriptional silencer, it is now known to have more complex effects on gene transcription, particularly on nucleosome positioning across regulatory elements and controlling access of DNA-binding proteins to enhancers and promoters [18]. The complex combines the enzymatic activities of HDAC1, HDAC2 and CHD4, together with chaperone proteins Rbbp4 and 7, zinc-finger proteins Gatad2a or Gatad2b, MTA1 and 2, Cdk2ap1 and, finally, Mbd2/3 [19]. Of these, CHD4 is the largest multidomain component; it is

critically connected with the other components and plays an essential role in many diseases [20,21]. NuRD components have been reported to physically interact with a wide repertoire of transcription factors in a cell context-dependent manner [22]. Whereas the therapeutic importance of the NuRD complex is widely acknowledged, its role in both healthy tissue homeostasis and disease makes it a challenging drug target. As an example, pharmacological targeting of the NuRD complex has been shown to synergize with epigenetic drugs [23]. In another genomic study, NuRD components were shown to contribute differentially to globin regulation [21]. These examples demonstrate the therapeutic significance of the NuRD complex and underline its disease-state-specific role. Understanding the complex internal architecture of the NuRD complex together with its context-dependent transcriptional activity remains a challenge and encourages further studies [24].

Deciphering the structural makeup of the NuRD complex and elucidating its vulnerabilities are of paramount therapeutic importance. Recent advances in CRISPR-based mutagenesis provide unprecedented opportunity for genomic studies to identify disease-relevant druggable sites within the NuRD complex. We present here a case study where we used CETSA to measure proteomic changes caused by systematic CRISPR mutagenesis of the NuRD complex and we demonstrate the importance of this method for the identification of druggable sites. For the purpose of demonstration, we focused on CHD4, as it represents a large multidomain protein of critical therapeutic importance.

MATERIALS & METHODS

Generation of stable clones

Stable Cas9-expressing HUDEP-2 cells were prepared as described before [21,25]. After confirmation of Cas9 expression using western blot analysis, cells were infected with lentivirus-carrying single sgRNAs to produce the stable clones F2 (CHD4 het), B8, B1 (CHD4 in-frame), B2 (CHD4 in-frame), A2 and D3 [21]. These clones have been shown to carry therapeutically relevant mutations of CHD4. Clone F2 represented wild-type CHD4 and the other clones carried various in-frame deletions – B1 (3 bp), B2 (9 bp), B8 (3 bp), A2 (3 bp) and D3 (3 bp) – in various domains of CHD4. The corresponding sgRNAs are described in Supplementary Table 1. Mutations were confirmed with sequencing and then HUDEP-2 clones were expanded, cultured and frozen for CETSA analysis. Procedures for cultivation, lentivirus production, selection and expansion of HUDEP-2 cells, as well as sequences of sgRNA, have been previously described [21].

CETSA analysis

Thermal profiles of NuRD proteins were identified in a temperature gradient using established methods [10,11]. Lysates were prepared from 10^6 cells for the six different HUDEP-2 clones and then western blot analysis was performed to measure the amount of remaining stable protein at each temperature point. For each of the clones, immunoblotting was performed to evaluate the effect of the mutations on the essential protein of the NuRD complex, particularly, CHD4, MTA2, GATAd2 and HDAC1. Band intensities were measured on LI-COR (NE, USA), then each protein was normalized to its corresponding counterpart in the F2 control and, subsequently, their corresponding thermal melting profiles were quantified. Further, differences in aggregation temperatures (T_{agg}) were calculated for each protein and presented as bar graphs. These experiments were repeated three-times for statistical power. All antibodies used in this study were purchased from Cell Signaling Technology (MA, USA).

The following optimizations were performed. Phosphate-buffered saline supplemented with 5% glycerol, 1% Triton™ X-100 and protease inhibitors (Roche, Basel, Switzerland) was used to lyse the cells under nondenaturing conditions to ensure that the protein content still retained its biophysical properties. The amount of cell lysate loaded into the SDS-PAGE gel was reduced to 5 μg per lane to ensure higher sensitivity of the melting profiles of the tested proteins and monoclonal antibodies were used for accurate determination of the melting profiles.

RESULTS & DISCUSSION

For the purpose of demonstration, we focused on CHD4 as it provides an ideally large and multidomain scaffold to support the NuRD complex. Using the CRISPR–Cas9 method, we generated several stable CHD4 mutants in HUDEP-2 cells. Subsequently, using CETSA, we investigated the stability of the NuRD complex, particularly of partner proteins CHD4, MTA2, GATAd2 and HDAC1, in each mutant. This was done in order to analyze perturbations caused by these in-frame mutations, potentially providing insight into therapeutic intervention against this complex. The focus of this method was to identify mutations that affected the thermal stability of the NuRD proteins. The effect of the mutations on the NuRD proteins was evaluated by CETSA (Figure 1A–D) and their corresponding thermal melting profiles were plotted (Figure 2A–D).

Initially, we measured the thermal melting pattern of CHD4 in these six clones. We found that, in comparison with the clone F2, all of the clones had a stabilizing effect on CHD4, with clones B2, B1, D3 and B8 having $\Delta T_{agg}(50)$ values of 1.1, 4.1, 6.9 and 4.8°C, respectively (Figures 1A & 2A). Subsequently, we measured the effect of the clones on the other NuRD proteins MTA2, GATAD2 and HDAC1. For MTA2, the clones A2 and B1 had a measurable destabilizing effect with $\Delta T_{agg}(50)$ values of -2.3 and 2.4°C, respectively, and clone D3 had a stabilizing effect with a $\Delta T_{agg}(50)$ of 5.0°C (Figures 1B & 2B). For GATAD2, all of the clones had a destabilizing effect with $\Delta T_{agg}(50)$ values of -5.0, -4.9, -2.5, -1.3 and -0.7°C for clones A2, B2, B1, D3 and B8, respectively (Figures 1C & 2C). For HDAC1, the clone A2 had a destabilizing effect with a $\Delta T_{agg}(50)$ of -1.8°C and the clones D3 and B8 had a stabilizing effect with $\Delta T_{agg}(50)$ values of 5.3 and 2.3°C, respectively (Figures 1D & 2D). Differences in the thermal melting profiles of the NuRD proteins in each clone were plotted (Figure 3).

Tiling CRISPR is emerging as a comprehensive method to discover and investigate the vulnerabilities of protein complexes that are important in both health and disease. As with many experimental methods, there are pros and cons that will reflect on the quality of results. When working with novel protein complexes, it is recommended to perform unbiased pooled CRISPR screens. Such an approach will explore all essential positions inside the complex and will provide a comprehensive map of vulnerabilities; however, for certain applications, it may be preferable to investigate the therapeutic potential of a limited number of defined positions in a protein complex. For this reason, we inves- ▶

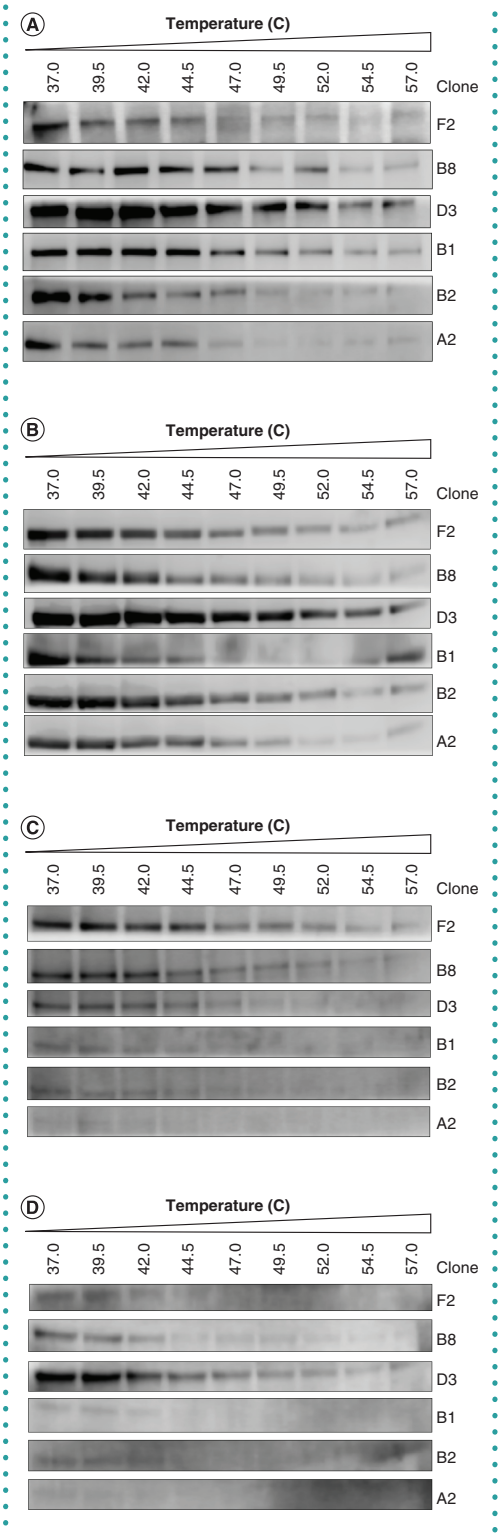


Figure 1. Immunoblotting images of the selected NuRD proteins. (A) CHD4; (B) MTA2; (C) GATA2D; (D) HDAC1. Six clones were analyzed and compared with the wild-type control, F2. The abundance of stable protein fractions was measured at each point of the heat gradient (n = 3). Band intensities were measured on LI-COR, normalized to its corresponding counterpart in F2 control.

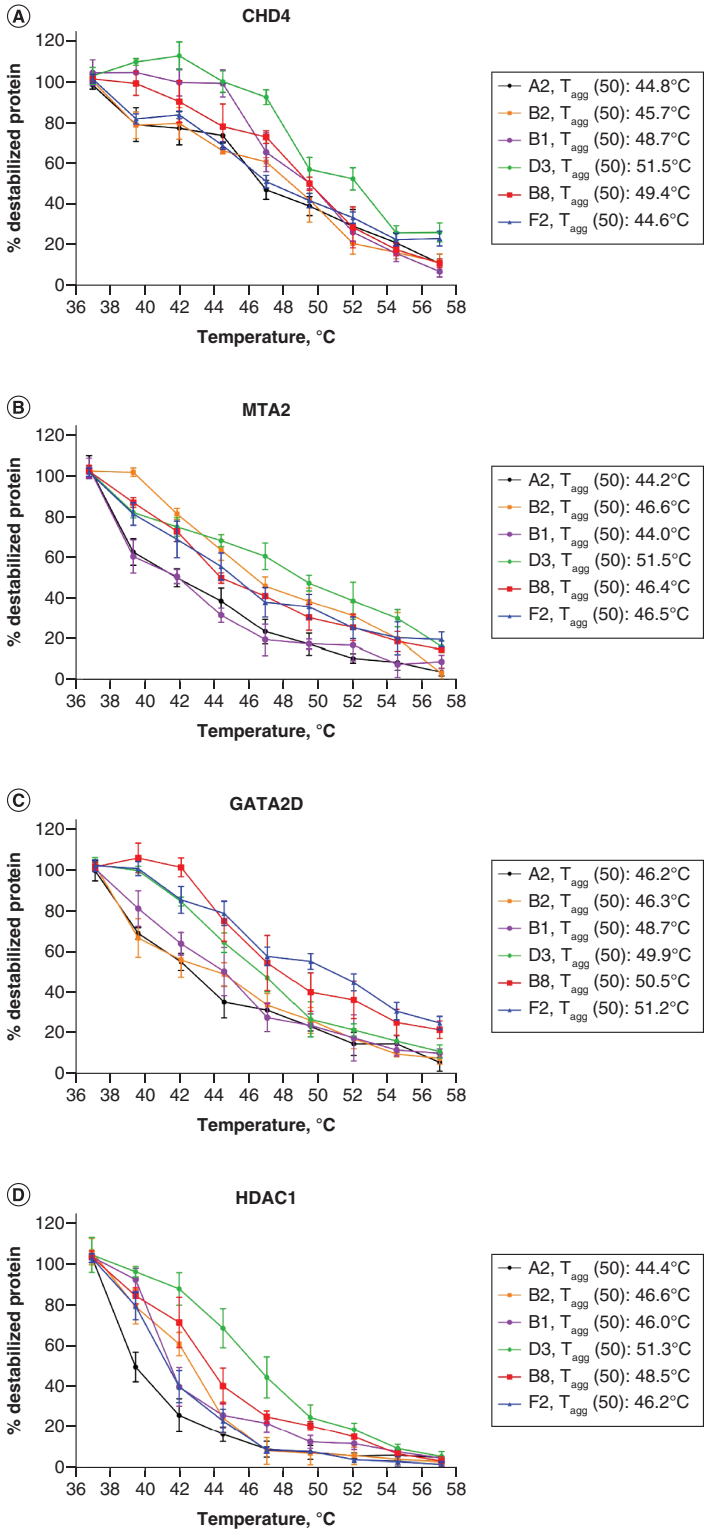
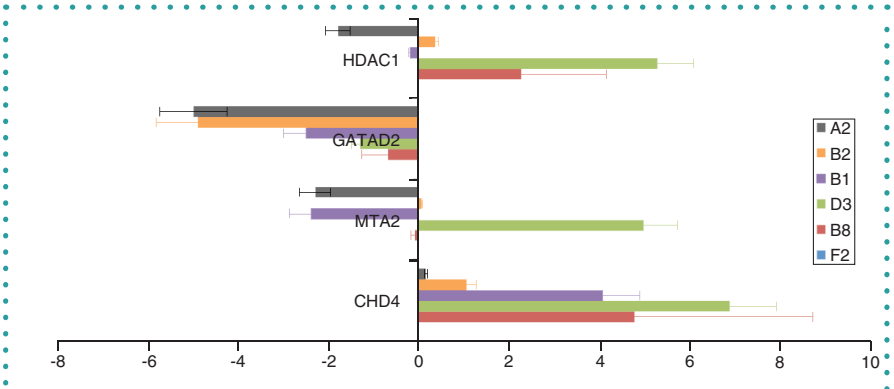


Figure 2. Thermal melting curves of the selected NuRD proteins. (A) CHD4; (B) MTA2; (C) GATA2D; (D) HDAC1. The curves were derived from the quantitated immunoblotting analyses shown in Figure 1. Six clones were analyzed and compared with the wild-type control, F2. The abundance of stable protein fractions was measured at each point of the heat gradient (n = 3) and then T_{agg}(50) was calculated for each protein.



	CHD4	MTA2	GATAD2	HDAC1
A2	0.2	-2.3	-5	-1.8
B2	1.1	0.1	-4.9	0.4
B1	4.1	-2.4	-2.5	-0.2
D3	6.9	5	-1.3	5.3
B8	4.8	-0.1	-0.7	2.3
F2	0	0	0	0

Δ Tagg (50), °C

Figure 3. Bar plot of the differences in temperature of aggregation, $T_{agg}(50)$, induced by mutations. The levels of stable protein from each clone were normalized to the corresponding protein from F2 control. Differences in $T_{agg}(50)$ were calculated and plotted as ΔT.

tigated the effect of only six CRISPR mutants on the NuRD complex.

For the purpose of this study, all of the selected clones carried short in-frame deletions, as these have practical utility for investigating their therapeutic effects. It should, however, be acknowledged that CRISPR screens could result in a variety of ensuing DNA outcomes per cleavage site, such as single mutations, deletions or insertions [26]. Although the most preferred type of DNA repair is point mutations for the purpose of determining individual amino acid positions of protein complexes, short and long indels (insertions or deletions) can also occur. Short in-frame indels are preferred to long frameshift and stop mutations, as the latter will completely destroy protein function. In this vein, the CRISPR library and the subsequent analysis should provide sufficient depth in order to exhaustively investigate all positions in a protein complex. This may translate into identification of therapeutically important sites that may otherwise not be well represented. These parameters may depend on the investigator's scientific vigor and hypotheses, which can be optimized using established methods [27,28]. Another point of concern related to CRISPR technology is unintended off-target effects. Especially for CRISPR-based medical therapies, scientists need to provide compelling documentation of potential off-target effects as these therapies advance toward clinical use. It is becoming acknowledged that, with well-designed nucleases, the mutation frequency for CRISPR should fall below the level of spontaneously occurring mutations [29]. Unlike biomedical applications of CRISPR where gene-corrected cellular materials can be used as therapies, off-target effects are not of measurable concern for drug discovery [30]. CRISPR technology can provide an initial systematic framework for investigating the importance of proteins as therapeutic targets, in addition to providing guidance to specific domains for subsequent targeting with drug molecules. CRISPR-modified cells can only be used as research tools in this rigorous discovery process, but the ultimate product is a drug molecule with potential clinical use that does not expose patients to risky cellular materials.

The ideal outcome of a tiling CRISPR screen is identification of mutations that mimic the binding of drug molecules. Such mutations may shift the stability of the carrying protein and, potentially, its partner proteins; however, within the complex proteomic context of the cell, effects of mutations may not be clearly discernible. Although a particular mutation may seem to have a stabilizing effect in one cell type, it might have an opposite effect in another cell line where the architecture of the investigated protein complex may differ. Similarly, not all mutations may translate into shifts in the thermal profiles of investigated proteins, depending on the cellular context and assay conditions. Therefore, all mutations triggering a shift in protein stability should be noted and studied individually in follow-up experiments within the selected cellular context.

A CRISPR screen paired with CETSA has the potential to decipher vulnerabilities inside therapeutically important protein complexes, such as the NuRD epigenetic complex. In this focused study using six CHD4 mutants, we determined their differential effects on the stability of other NuRD components, particularly MTA2, GATAD2 and HDAC1. HDAC1 provides the enzymatic activity necessary to epigenetically mark DNA and is the most structurally embedded component of the complex [31]. Therefore, one can speculate that mutations indirectly affecting the stability of HDAC1 might have the greatest therapeutic impact. In the context of this limited study, these mutants were D3 and B8. However, the effects of these mutations should be further investigated. It is also important to expand future studies to systematically mutate the other components to fully understand protein–protein interaction within the complex and to map their druggable vulnerabilities. In this study, we demonstrated that a combined CRISPR-CETSA methodology can be used to accelerate disease-relevant drug discovery.

FUTURE PERSPECTIVE

CRISPR technology has revolutionized drug discovery by providing a comprehensive framework from target validation to hit selection. The wealth of genomics data produced in this way must be efficiently linked to proteomics and the physiological state of the cell. The ensuing genome–proteome link will help build stronger hypotheses regarding therapeutically important protein–protein interactions and help identify proteomic vulnerabilities specific to disease states.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper, please visit the ▶

journal website at: www.future-science.com/doi/suppl/10.2144/btn-2019-0100

AUTHOR CONTRIBUTIONS

N-G Her designed the experiments and helped with manuscript preparation. I Babic and VM Yenugonda contributed to the scientific integrity of the study. S Kesari provided supervision and contributed to the scientific integrity of the study. E Nurmemmedov designed the study and contributed to manuscript preparation.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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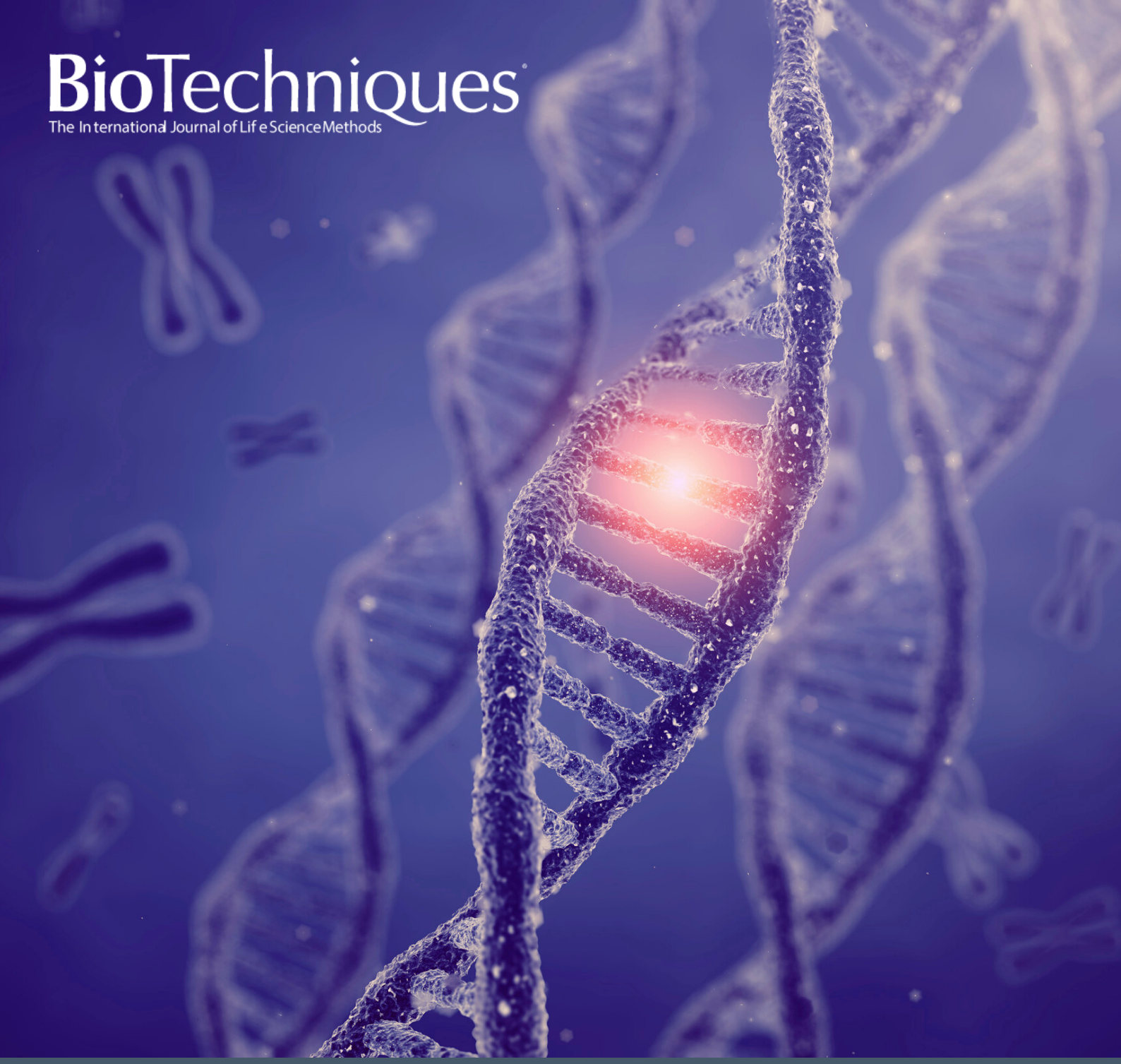

Contact us

Editorial Department

Senior Editor
Abigail Sawyer
asawyer@biotechniques.com

Business Development and Support

Commercial Director
Sarah Mayes
s.mayes@future-science-group.com

@MyBioTechniques @biotechniques BioTechniques

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